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Award Number: DAMD17-03-1-0610

TITLE: Functional Analysis of Chk2-Kiaa0170 Interaction

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REPORT DATE: September 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2006		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 15 AUG 2003 - 14 AUG 2006	
4. TITLE AND SUBTITLE Functional Analysis of Chk2-Kiaa0170 Interaction				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0610	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Zhenkun Lou, Ph.D. E-Mail: lou.zhenkun@mayo.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mayo Clinic and Foundation Rochester, MN 55905				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: Chk2 is a critical regulator of DNA damage repair checkpoint controls. Mutations in Chk2 confer an increased risk of breast cancer. However, the regulation of Chk2-mediated pathways is still not clear. We previously identified Kiaa0170 (later renamed MDC1, Mediator of DNA Damage Checkpoint Protein 1), interacts with Chk2 after DNA damage. We hypothesized that MDC1 is a key regulator of Chk2-dependent pathways, and is directly involved in DNA repair and cell cycle control. Dysfunction of MDC1-Chk2 pathway could be a key event in tumorigenesis. During funding period, we mapped the interaction sites responsible for the Chk2-MDC1 interaction (Task 1). We also show that MDC1 regulates Chk2-dependent cell cycle checkpoint and radiation-induced apoptosis (Task 2). Finally we generated MDC1 knockout mice and provide evidence at the organismal level that MDC1 is a critical regulator of the DNA damage response pathway and genomic stability (revised Task 3). MDC1 mice also show increased tumor incidence, suggesting that MDC1, like Chk2, is a potential tumor suppressor. Our work provide novel insight into the mechanism of the DNA damage response pathway and tumor suppression.					
15. SUBJECT TERMS Tumorigenesis, Chk2, MDC1, Genomic Stability, DNA Damage Response, Checkpoint Activation.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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Introduction

Chk2 is a critical regulator of DNA damage repair checkpoint controls. In response to DNA damage, Chk2 is activated and initiates DNA damage-signaling pathways by regulating downstream substrates, such as p53 and BRCA1. Like those of p53 and BRCA1, mutations in Chk2 confer an increased risk of breast cancer. However, the regulation of Chk2-mediated pathways is still not clear. Identification of proteins that associate with Chk2 will provide new clues to the regulation of Chk2-dependent pathways. Based on our preliminary studies that Kiaa0170 (late renamed MDC1 (Mediator of DNA Damage Checkpoint Protein 1)) interacts with Chk2 following DNA damage, we hypothesized that MDC1 regulates Chk2 function and is directly involved in DNA repair and cell cycle control. Dysfunction of MDC1-Chk2 pathway could be a key event in tumorigenesis. We proposed to study the functional interaction between MDC1 and Chk2. Since Chk2 is an important regulator of the DNA damage response pathway and a tumor suppressor, study the MDC1-Chk2 interaction might shed new light in the mechanism of DNA damage response and tumor suppression.

Body

In the statement of work we proposed to study the following tasks:

Task1. Characterize the physical interaction between MDC1 and Chk2

Task2. Investigate the functional interaction of MDC1 and Chk2 in DNA damage response pathway

Task 3. (Revised) Use MDC1^{-/-} cells to study the in vivo function of MDC1

From Aug15, 2003-Aug14, 2006, we performed following studies:

Task1. Characterize the physical interaction between MDC1 and Chk2

We performed immunofluorescence of MDC1 and Chk2. While MDC1 and Chk2 diffusely localize in the nucleus in untreated cell, following DNA damage, Both MDC1 and Chk2 colocalize to discrete nuclear foci, representing the sites of DNA damage (see Figure 2B, Appendix 1, Nature 421: 957-961). In addition, by performing GST-pull down assay, site-directed mutagenesis, and peptide competition assay, we have mapped the sites of interaction between MDC1 and Chk2. We show that the FHA domain of MDC1 interacts with Thr68 of Chk2 in a phosphorylation dependent manner (see Figure 2d-2h, Appendix 1, Nature 421: 957-961). Since Thr68 of Chk2 is phosphorylated following DNA damage, this result is consistent with the observation that MDC1 interacts with Chk2 following DNA damage.

Task2. Investigate the functional interaction of MDC1 and Chk2 in DNA damage response pathway

MDC1 contributes to Chk2 activation

We further investigated the functional significance of the MDC1-Chk2 interaction by examining how MDC1 affects Chk2 function. To do this, we synthesized siRNAs to downregulate MDC1. The siRNAs effectively downregulate MDC1 when transfected into HeLa or A549 cells. We next examined the activation of Chk2 and Chk2 foci formation following DNA damage when MDC1 was downregulated. We failed to detect any defect in Chk2 phosphorylation and foci formation in cells transfected with MDC1 siRNA (data not shown). However, we did observe a defect in Chk2 phosphorylation following low dose of radiation in MDC1^{-/-} cells (see Figure 6B, Appendix 3, Mol. Cell 21:1-14). These results suggest that MDC1, although not required for Chk2 activation, contributes to Chk2 activation.

MDC1 regulates Chk2-dependent checkpoint activation and radiation-induced apoptosis.

We next examined how MDC1 regulates Chk2-dependent functions. We show that intra-S phase checkpoint and radiation-induced apoptosis become defective in cells transfected with MDC1 siRNA, suggesting that MDC1 regulates Chk2-dependent checkpoint activation and apoptosis (see Figure 3, Appendix 1, Nature 421: 957-961). In addition, we provide evidence that the MDC1-Chk2 interaction is required for these Chk2-dependent functions (see Figure 4, Appendix 1, Nature 421: 957-961).

MDC1 regulates BRCA1 foci formation and phosphorylation

We also investigated how MDC1 regulates BRCA1, which has been shown to act downstream of Chk2. We show that MDC1 is required for both BRCA1 foci formation and phosphorylation following DNA damage. These results suggest that MDC1 is an upstream regulator of BRCA1 (see Appendix 2, J. Biol. Chem 278:13599-13603).

Overall, our results suggest that MDC1 is an important regulator of Chk2-dependent pathway.

Task 3. (Revised) Use MDC1^{-/-} cells to study the in vivo function of MDC1

MDC1 maintains genomic stability

Since Task1 and Task 2 are performed in cancer cell lines, which already contain a certain degree of genomic instability, it is difficult to examine the role of MDC1 in maintaining genomic stability. We took splenocytes from MDC1^{-/-} mice and examined whether MDC1^{-/-} cells display genomic instability. 17.5% MDC1^{-/-} cells display various degrees of chromosome aberrations, including chromosome breaks, chromatid breaks, and chromosome fusion (see Figure 3E, Appendix 3, Mol. Cell 21:1-14). In contrast, MDC1^{+/+} cells display few such chromosome aberrations. These results suggest that MDC1 plays an important role in maintaining genomic stability.

MDC1 participates in DNA repair

We also evaluated the DNA repair capability of MDC1. We took primary mouse embryonic fibroblasts (MEFs) from MDC1^{+/+} or MDC1^{-/-} mice. These cells were irradiated and then let recover from DNA damage. MDC1^{+/+} cells repaired DNA damage effectively, however, MDC1^{-/-} cells were inefficient in repairing DNA damage (see Figure 3C-D, Appendix 3, Mol. Cell 21:1-14). These results suggest that MDC1 participates in NDA repair.

MDC1 regulates the recruitment of DNA damage factors

We also found that in the absence of MDC1, the accumulation of DNA damage factors (foci formation) such as active ATM, 53bP1, NBS1, at the sites of DNA damage become defective (See Figure 4C, Figure 6A, Appendix 3, Mol. Cell 21:1-14). These results suggest that MDC1 is important for the accumulation of DNA damage factors to the sites of DNA damage, thereby facilitating DNA repair. Since the accumulation of active ATM, 53bP1, NBS1 at the sites of DNA damage is independent of Chk2, these results suggest MDC1 also functions in Chk2-independent pathway.

MDC1 regulates DNA damage checkpoint activation

We have shown that MDC1 regulates DNA damage checkpoint activation in Task 2 using siRNA to downregulate MDC1 in cancer cell lines. Here we use MDC1^{-/-} cells to further examine the role of MDC1 in DNA damage checkpoint activation. We show that MDC1^{-/-} cells have defective Chk1 and Chk2 activation, resulting defective checkpoint activation following DNA damage (Figure 6, Appendix 3, Mol. Cell 21:1-14). These results confirmed our results in Task 2.

MDC1^{-/-} cells show radiosensitivity

Since MDC1^{-/-} cells show both defective checkpoint activation and DNA repair, they should be vulnerable to DNA damage. Indeed, MDC1^{-/-} cells show hypersensitivity to radiation (Figure 3B, Appendix 3, Mol. Cell 21:1-14). These results suggest that MDC1 is critical for the DNA damage response.

Key Research Accomplishments

- * The MDC1 FHA domain interacts with phospho-The68 of Chk2
- * MDC1 contributes to Chk2 activation
- * MDC1 regulates Chk2-dependent checkpoint activation and radiation-induced apoptosis.
- * MDC1 regulates BRCA1 foci formation and phosphorylation
- *MDC1 regulates the recruitment of DNA damage factors
- *MDC1 participates in DNA repair
- * MDC1 maintains genomic stability
- *MDC1^{-/-} cells show radiosensitivity

Reportable Outcomes

Publications:

1. Lou, Z., Minter-Dykhouse, K., Wu, X. and Chen, J. MDC1 Is Coupled to Activated Chk2 in Mammalian DNA Damage Response Pathways. *Nature* 421:957-961, 2003.
2. Lou, Z., Chini, C., Minter-Dykhouse, K., and Chen, J. MDC1 Regulates BRCA1 Relocalization and Phosphorylation in DNA damage Checkpoint. *J. Biol. Chem.*, 278:13599-13603, 2003.
3. Lou, Z and Chen, J. BRCA proteins and DNA Damage Checkpoints. *Frontiers in Bioscience*. 8:S718-721, 2003.
4. Lou, Z, Chen, B. P-C., Asaithamby, A., Minter-Dykhouse, K., Chen, D.J. and Chen, J. MDC1 Regulates DNA-PK Autophosphorylation in Response to DNA Damage. *J. Biol. Chem.* 279:46359-62, 2004.
5. Lou, Z., Minter-Dykhouse, K, Junjie Chen. BRCA1 Participates In DNA Decatenation. *Nature Structure and Molecular Biology*, 12:589-563, 2005.
6. Lou, Z, et al. MDC1 Maintains Genomic Stability by Participating in the Amplification of ATM-Dependent DNA Damage Signals. *Molecular Cell*, 21:1-14, 2006.
7. Franco, S., Gostissa, M., Zha, S., Lombard, D.B., Murphy, M.M., Zarrin, A.A., Yan, C., Tepsuporn, S., Morales, J.C., Adams, Lou, Z., Bassing, C.H., Manis, J.P., Chen, J., Carpenter, P.B., and Alt, F.W. H2AX Prevents DNA Breaks From

Progressing To Chromosome Breaks and Translocations. **Molecular Cell, 21:201-14, 2006**

8. Lou, Z and Chen, J. Cellular senescence and DNA repair. **Experimental Cell Research. 312:2641-2646, 2006.**

Book Chapters

1. Lou, Z and Chen, J. **Methods In Molecular Biology** (Human Press) : Use of siRNA to study the function of MDC1 in DNA damage responses. **Methods in Molecular Biology, 281(2): 179-187. Humana Press.**
2. Lou, Z and Chen, J. Mammalian DNA Damage Response Pathway. **In Press. Genome Instability in Cancer Development. Springer Press.**

Grant Applied

Based on my findings, my mentor Dr. Junjie Chen successfully applied a RO1 grant: "Study the role of DNA damage responses in tumorigenesis and senescence" Grant Number: 2R01CA092312-06, from NCI.

Employment opportunities:

Based on training supported by this award, I obtained a tenure-track assistant professor position at Mayo Clinic, Division of Oncology Research, in April 2006.

Conclusion:

In summary, our findings suggest the MDC1 is an important mediator of the DNA damage response pathway. MDC1 regulates both Chk2-dependent and Chk2-independent pathways. The loss of MDC1 results in genomic instability and defective DNA damage response. Our results reveal a novel player of the DNA damage response pathway and shed new light on the mechanism of DNA damage response and tumor suppression.

Appendices

1. Lou, Z., Minter-Dykhouse, K., Wu, X. and Chen, J. MDC1 Is Coupled to Activated Chk2 in Mammalian DNA Damage Response Pathways. **Nature 421:957-961, 2003.**
2. Lou, Z., Chini, C., Minter-Dykhouse, K., and Chen, J. MDC1 Regulates BRCA1 Relocalization and Phosphorylation in DNA damage Checkpoint. **J. Biol. Chem., 278:13599-13603, 2003.**
3. Lou, Z., et al. MDC1 Maintains Genomic Stability by Participating in the Amplification of ATM-Dependent DNA Damage Signals. **Molecular Cell, 21:1-14, 2006.**
4. **Bibliography**

MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways

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Forkhead-homology-associated (FHA) domains function as protein–protein modules that recognize phosphorylated serine/threonine motifs^{1–5}. Interactions between FHA domains and phosphorylated proteins are thought to have essential roles in the transduction of DNA damage signals; however, it is unclear how FHA-domain-containing proteins participate in mammalian DNA damage responses. Here we report that a FHA-domain-containing protein—mediator of DNA damage checkpoint protein 1 (MDC1; previously known as KIAA0170)—is involved in DNA damage responses. MDC1 localizes to sites of DNA breaks and associates with CHK2 after DNA damage. This association is mediated by the MDC1 FHA domain and the phosphorylated Thr 68 of CHK2. Furthermore, MDC1 is phosphorylated in an ATM/CHK2-dependent manner after DNA damage, suggesting that MDC1 may function in the ATM–CHK2 pathway. Consistent with this hypothesis, suppression of MDC1 expression results in defective S-phase checkpoint and reduced apoptosis in response to DNA damage, which can be restored by the expression of wild-type MDC1 but not MDC1 with a deleted FHA domain. Suppression of MDC1 expression results in decreased p53 stabilization in response to DNA damage. These results suggest that MDC1 is recruited through its FHA domain to the activated CHK2, and has a critical role in CHK2-mediated DNA damage responses.

The DNA-damage-induced cell-cycle checkpoints and apoptosis are critical for the maintenance of genomic stability^{6,7}. Many proteins involved in DNA damage responses contain functional domains such as FHA and BRCA1 carboxy-terminal (BRCT) domains^{1,8}. FHA and BRCT domains are molecular modules involved in protein–protein interaction. FHA domains specifically recognize motifs that contain phosphorylated serine/threonine in a manner similar to the SH2-phosphotyrosine interaction^{2–5}. A well-studied example of the FHA-domain-mediated interaction is the Rad9–Rad53 interaction in *Saccharomyces cerevisiae*^{9–11}, which is critical for the activation of DNA damage pathways in yeast. However, except for the homo-oligomerization of checkpoint kinase 2 (CHK2)^{12,13}, such FHA-domain-mediated interactions are not documented in mammalian cells. More importantly, the role of FHA domains in DNA damage signal transduction remains to be established in mammals.

MDC1 is a protein that contains both FHA and BRCT domains. Because the BRCT domain of MDC1 shares close sequence homology with that of BRCA1 and yeast Rad9, we examined whether MDC1 is involved in DNA damage response pathways in mammalian cells. Many proteins involved in DNA damage response pathways form nuclear foci after DNA damage (for example, BRCA1 and CHK2)^{14,15}. Therefore, we first investigated the nuclear localization of MDC1 before and after DNA damage. MDC1 normally localized in the nuclei without any apparent structures. However, after γ -irradiation, MDC1 relocated to nuclear foci (Fig. 1a) and localized together with phosphorylated H2AX (γ H2AX) foci, suggesting that MDC1 relocates to the sites of DNA damage^{16,17}. In addition, similar to many other signalling proteins in DNA damage response pathways, MDC1 migrates slower after γ -irradiation (Fig. 1b). Treatment of MDC1 immunoprecipitates with λ -phosphatase reverses this mobility shift (Fig. 1b), suggesting

that MDC1 is phosphorylated after γ -irradiation. To determine whether the phosphorylation of MDC1 depends on ataxia–telangiectasia mutated (ATM), K562 cells and ATM-deficient GM03189D cells were either irradiated or left untreated. In contrast to that in K562 cells, no mobility shift of MDC1 was observed in GM03189D cells after γ -irradiation (Fig. 1c), suggesting that the phosphorylation of MDC1 is ATM-dependent.

To explore the role of MDC1 in DNA damage pathways, we examined whether MDC1 would associate with proteins involved in DNA damage response pathways. As shown in Fig. 2a, MDC1 associated with CHK2 only after DNA damage. This interaction is not interrupted by ethidium bromide (data not shown), suggesting that the MDC1–CHK2 interaction is not mediated by DNA. Furthermore, MDC1 localized together with the phosphorylated Thr 68 residue of CHK2 after DNA damage (Fig. 2b). As MDC1 interacts with the active form of CHK2 (Thr-68-phosphorylated CHK2) after DNA damage, we investigated whether the phosphorylation of MDC1 requires CHK2. CHK2-deficient HCT15 cells and HCT15 cells stably transfected with either wild-type CHK2 or kinase-dead CHK2 were irradiated or left untreated. No mobility shift of MDC1 was observed in HCT15 cells after γ -irradiation. However, mobility shift of MDC1 was restored in HCT15 cells reconstituted with wild-type CHK2, but not in cells reconstituted with kinase-dead CHK2 (Fig. 2c).

To explore further the interaction between MDC1 and CHK2, we generated glutathione S-transferase (GST) fusion proteins that contained either the MDC1 FHA domain or the MDC1 BRCT domain, as FHA and BRCT domains are involved in protein–protein interactions. Figure 2d shows that GST–MDC1(FHA), but not GST–MDC1(BRCT), interacted with CHK2 after DNA damage. The *in vitro* interactions of GST–MDC1(FHA) and CHK2 are

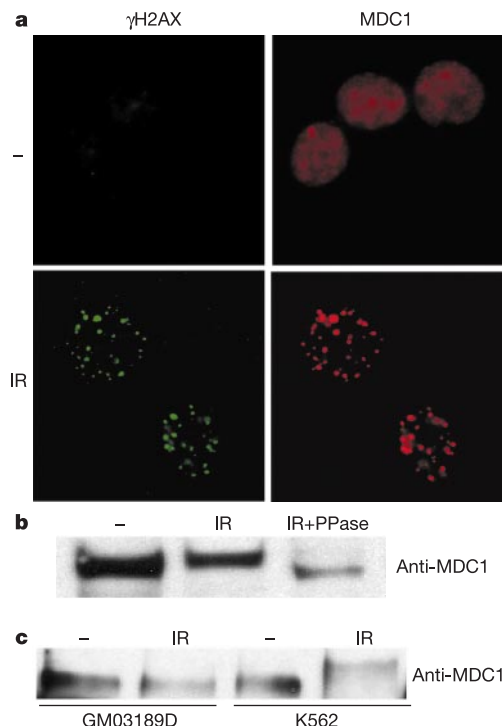


Figure 1 MDC1 is phosphorylated and relocates to nuclear foci after DNA damage. **a**, 293T cells were left untreated (top panels) or treated with 10 Gy γ -irradiation (IR; bottom panels), and 10 h later they were stained with anti- γ H2AX antibodies (left) or anti-MDC1 antibodies (right). **b**, MDC1 was immunoprecipitated from untreated or γ -irradiated (30 Gy) K562 cells and incubated with buffer or λ -phosphatase (PPase), then blotted with anti-MDC1 antibodies. **c**, K562 cells and GM03189D cells (ATM deficient) were γ -irradiated (30 Gy) or left untreated, then blotted with anti-MDC1 antibodies.

specific and depend on the integrity of the FHA domain of MDC1, as mutations in highly conserved residues within this domain (R58A, S72A, N96G97T98/AAA) abolished or reduced the interaction between MDC1 and CHK2 (data not shown). To test whether the MDC1 FHA domain is required for the MDC1–CHK2 interaction *in vivo*, we generated constructs encoding S-tagged wild-type MDC1 or MDC1 with a deleted FHA domain (MDC1 Δ FHA). 293T cells transfected with these constructs were irradiated and lysates were incubated with S-Sepharose to pull-down wild-type or the deletion mutant of MDC1. As shown in Fig. 2e, in contrast with wild-type MDC1, MDC1 Δ FHA failed to interact with CHK2. These results suggest that the damage-dependent interaction between MDC1 and CHK2 depends on the FHA domain of MDC1.

To examine which region of CHK2 is important for the MDC1–CHK2 interaction, constructs encoding haemagglutinin (HA)-tagged wild-type CHK2 (CHK2WT) and CHK2 deleted of the S/TQ-rich region (CHK2 Δ S/TQ) were stably transfected into CHK2-deficient HCT15 cells. Deletion of the S/TQ-rich region of CHK2 completely abolished the MDC1–CHK2 association, suggesting that the S/TQ-rich region of CHK2 is required for MDC1 binding (data not shown). The S/TQ-rich region of CHK2 contains multiple potential phosphorylation sites for ATM/ataxia–telangiectasia and Rad3 related (ATR) kinases. Among them, Thr 68 has been shown to be an ATM phosphorylation site that is critical for CHK2 activation^{18–22}. Notably, as determined by degenerate peptide library studies, the CHK2 sequence surrounding phosphorylated Thr 68 is a predicated recognition sequence of the MDC1 FHA domain⁴. Therefore, we next tested whether phosphorylated Thr 68 of CHK2

is the site recognized by the MDC1 FHA domain. Again, *in vitro* pull-down assay using GST–MDC1(FHA) was performed using HCT15 cells stably transfected with CHK2WT, kinase-dead CHK2 (CHK2KD) or CHK2 containing a T68A mutation (CHK2T68A). Whereas the kinase-dead mutant of CHK2 still interacted with MDC1 in a DNA-damage-dependent manner (Fig. 2f), the T68A mutation abolished the MDC1–CHK2 interaction. These results suggest that the phosphorylation of CHK2 at Thr 68 by ATM, but not CHK2 autophosphorylation sites, is critical for MDC1 binding.

To confirm further the critical role of Thr-68-phosphorylated CHK2 in the MDC1–CHK2 interaction, synthetic CHK2 peptides representing CHK2 amino acid sequence surrounding Thr 68 (residues 64–73) were prepared with Thr 68 as either a phosphorylated (p-T68) or non-phosphorylated (T68) residue. If phosphorylated Thr 68 and the surrounding sequence of CHK2 are truly recognized by the MDC1 FHA domain, there would be competition between the p-T68 peptide and endogenous CHK2 for binding to GST–MDC1(FHA). As expected, p-T68 peptide, but not T68 peptide, effectively blocked the binding of endogenous CHK2 to GST–MDC1(FHA) after DNA damage (Fig. 2g). As a negative control, a phosphorylated peptide containing CHK2 Ser 516 (an autophosphorylation site; our own unpublished observation) did not block the binding of CHK2 with MDC1 (Fig. 2g). Furthermore, when p-T68 or T68 peptides were conjugated to Sepharose and incubated with cell lysates, only p-T68 peptide was able to interact with MDC1 (Fig. 2h). To demonstrate the direct interaction between the MDC1 FHA domain and the phosphorylated Thr 68 site of CHK2, T68 or p-T68 peptide conjugated with Sepharose were

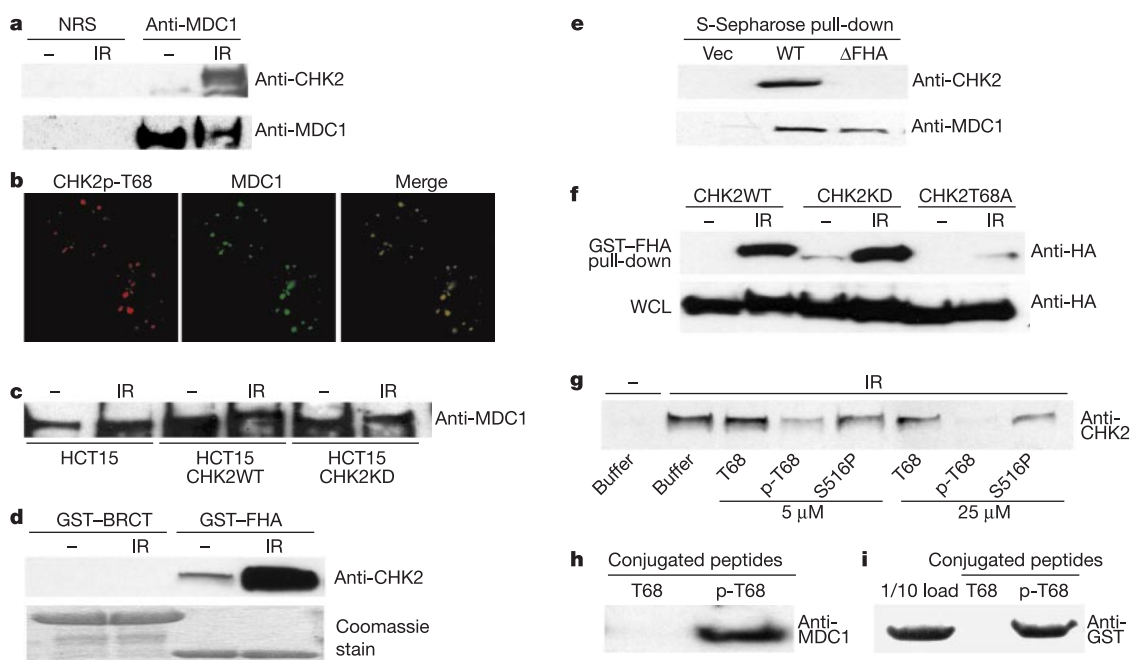


Figure 2 MDC1 interacts with CHK2 through the MDC1 FHA domain and the phosphorylated Thr 68 of CHK2. **a**, Cell lysates from K562 cells untreated or treated with 30 Gy γ -irradiation were subjected to immunoprecipitation with normal rabbit serum (NRS) or anti-MDC1 antibodies, and the immunoprecipitates were analysed with anti-CHK2 antibody. **b**, 293T cells were irradiated (10 Gy) and 10 h later stained with the indicated antibodies. **c**, HCT15 cells (CHK2 deficient) and HCT15 cells stably transfected with wild-type CHK2 or kinase-dead CHK2 were γ -irradiated (30 Gy) or left untreated, and cell lysates were then blotted with anti-MDC1 antibodies. **d**, *In vitro* binding assay. Beads bound with GST–MDC1(FHA) and GST–MDC1(BRCT) were incubated with lysates from control or γ -irradiated (30 Gy) K562 cells. Binding of CHK2 was analysed by immunoblotting. **e**, 293T cells were transfected with the indicated constructs and treated with 30 Gy γ -irradiation. The association of S-tagged MDC1 with CHK2 was assessed

using the indicated antibodies. WT, wild type. **f**, *In vitro* binding assays. HCT15 cells stably transfected with the indicated HA-tagged constructs were treated with 30 Gy γ -irradiation or left untreated. Beads bound with GST–MDC1(FHA) were used to pull-down HA-tagged wild-type or mutants, and were blotted with anti-HA antibodies. Whole cell lysates (WCL) were blotted with anti-HA antibodies (bottom panel). **g**, Peptide competition assay. K562 cell lysates were incubated with beads bound with GST–MDC1(FHA) plus the indicated concentration of CHK2 peptides. The remaining CHK2 on the beads was detected by anti-CHK2 antibodies. **h**, **i**, Non-phosphorylated or phosphorylated Thr peptides (T68 and or p-T68, respectively) conjugated to Sepharose beads were incubated with the lysate prepared from untreated K562 cells (**h**), or purified GST–MDC1(FHA) (**i**). Proteins bound on beads were analysed with anti-MDC1 antibodies (**h**) or anti-GST antibodies (**i**).

incubated with purified GST-MDC1(FHA). As shown in Fig. 2i, only p-T68 peptide strongly binds to the MDC1 FHA domain. These data suggest that phosphorylated Thr 68 of CHK2 acts as a docking site for MDC1.

The interaction of MDC1 and CHK2 after γ -irradiation suggests

that MDC1 may be involved in CHK2-mediated DNA damage responses. To investigate whether MDC1 functions in the ATM-CHK2 pathway, we used the small interfering RNA (siRNA) technique to suppress MDC1 expression²³. Transfection of MDC1 siRNA resulted in the suppression of MDC1 in A549 cells (Fig. 3a, b).

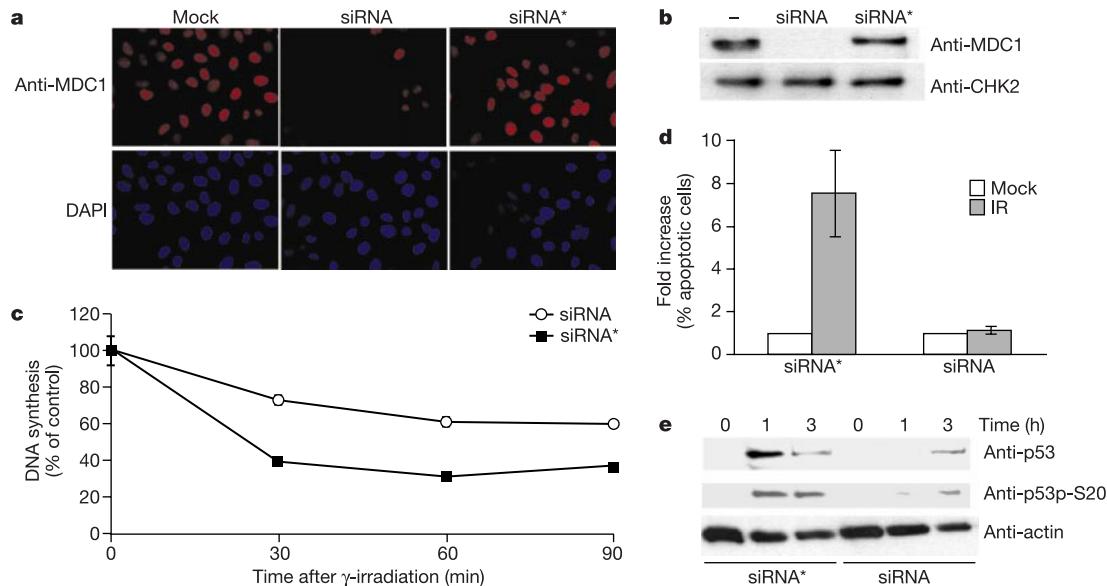


Figure 3 MDC1 is required for CHK2-mediated DNA damage responses. **a, b**, A549 cells were mock-transfected or transfected with MDC1 siRNA or control siRNA (siRNA*) twice. Seventy-two hours after the initial transfection, cells were immunostained with anti-MDC1 antibodies (**a**) or blotted with anti-MDC1 antibodies or CHK2 antibodies as a control (**b**). DAPI, 4,6-diamidino-2-phenylindole. **c**, A549 cells transfected with MDC1 siRNA or control siRNA as described for **a** and **b**. Cells were γ -irradiated (20 Gy) or left untreated,

and DNA synthesis was assessed. **d**, A549 cells were transfected with either control siRNA or MDC1 siRNA, then left untreated or irradiated (5 Gy). Thirty-six hours later, cells were collected and evaluated for apoptosis. **e**, A549 cells were transfected with either control siRNA or MDC1 siRNA, then irradiated (5 Gy). Cells were lysed at the time indicated and blotted for p53, Ser-20-phosphorylated (p-S20) p53 or actin.

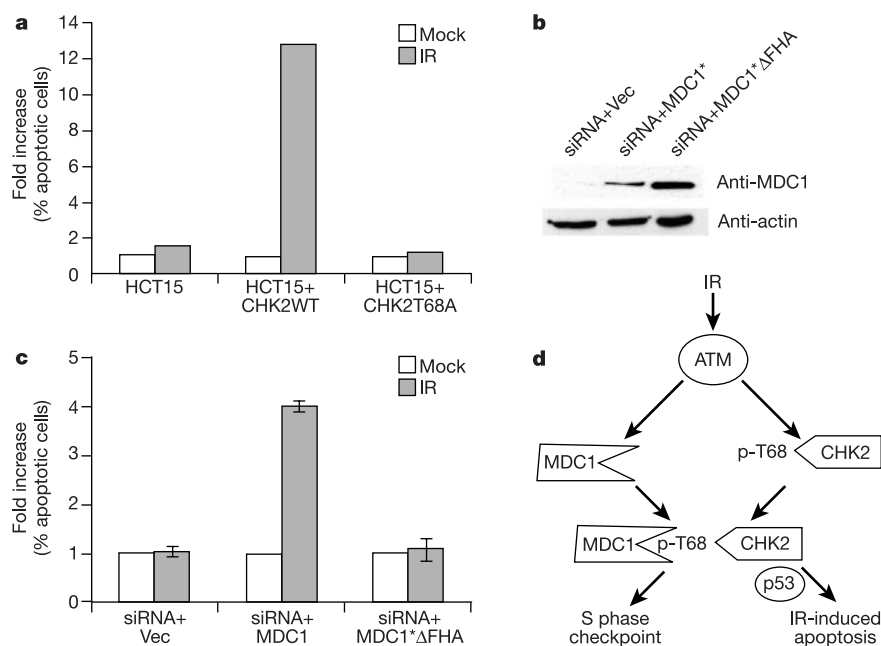


Figure 4 The functional interaction of CHK2 and MDC1 is required for CHK2-mediated DNA damage responses. **a**, HCT15 cells (CHK2 deficient) and HCT15 cells stably transfected with wild-type CHK2 or CHK2T68A were γ -irradiated (10 Gy) or left untreated. Thirty-six hours later, cells were collected and evaluated for apoptosis. **b, c**, A549 cells were transfected with MDC1 siRNA, and 24 h later they were transfected with constructs encoding green fluorescent protein (GFP) and either pCDNA3 vector, pCDNA3 encoding

siRNA-resistant wild-type MDC1 (MDC1*) or MDC1ΔFHA (MDC1*ΔFHA). Thirty-six hours after the initial transfection, cells were collected and blotted for MDC1 expression (**b**), or sorted for GFP-positive cells (**c**). The GFP-positive cells were then either irradiated (5 Gy) or left untreated, and 48 h later, cells were evaluated for apoptosis. **d**, Model of how MDC1 interacts with activated CHK2 and functions in CHK2-mediated DNA damage responses.

Similar effects were observed in HeLa cells, BJ cells and 293 T cells (data not shown). We also prepared control siRNA by introducing four point mutations into MDC1 siRNA (siRNA^{*}). Transfection of control siRNA did not affect the level of MDC1, indicating the specificity of MDC1 siRNA (Fig. 3a, b). To examine whether suppression of MDC1 expression affects CHK2-mediated DNA damage responses, we examined downstream events regulated by CHK2. One such event is the ATM/CHK2-dependent temporal inhibition of DNA synthesis in S phase^{24,25}. In ATM-deficient and CHK2-deficient cells, radioresistant DNA synthesis (RDS) occurs owing to the defective S-phase checkpoint^{24,25}. We examined DNA synthesis after γ -irradiation in cells transfected with MDC1 siRNA or control siRNA. There was a 60–70% inhibition of DNA synthesis in cells transfected with control siRNA after γ -irradiation (Fig. 3c). However, in cells transfected with MDC1 siRNA, there was only 30–40% inhibition, consistent with an RDS phenotype (Fig. 3c). Similar RDS phenotype was also observed in BJ cells transfected with MDC1 siRNA (data not shown). To evaluate further the role of MDC1 in CHK2-mediated DNA responses, we evaluated the radiation-induced apoptosis. Recent studies suggest that the radiation-induced apoptosis requires CHK2 activity^{26–28}. As shown in Fig. 3d, γ -irradiation induced apoptosis in A549 cells transfected with control siRNA. This γ -irradiation-induced apoptosis was abolished when A549 cells were transfected with MDC1 siRNA. These results indicate that MDC1 has a critical involvement in CHK2-mediated DNA damage responses. To explore further downstream molecules regulated by MDC1, we examined p53 stabilization after γ -irradiation, as CHK2 regulates radiation-induced apoptosis by phosphorylating p53 at Ser 20, which leads to the stabilization of p53 (refs 26–30). Compared with cells transfected with control siRNA, cells transfected with MDC1 siRNA show decreased and delayed p53 stabilization after γ -irradiation (Fig. 3e). Consistent with p53 stabilization, phosphorylation of p53 at Ser 20 is also decreased and delayed in cells transfected with MDC1 siRNA (Fig. 3e). These results indicate that MDC1 was able to regulate γ -irradiation-induced apoptosis by influencing p53 stabilization.

To establish specifically that the CHK2–MDC1 interaction is required for the DNA damage responses shown above, we evaluated γ -irradiation-induced apoptosis in HCT15 cells stably transfected with wild-type CHK2 or CHK2 with a mutation at Thr 68 (T68A), which could not associate with MDC1. As shown in Fig. 4a, HCT15 cells show defective apoptosis in response to γ -irradiation, which was restored by the reconstitution of wild-type CHK2, but not CHK2T68A. Furthermore, we generated MDC1 constructs (full-length and Δ FHA) that are resistant to siRNA targeting by introducing silent mutations at the region targeted by siRNA. Co-transfection of these constructs with MDC1 siRNA can restore the expression of MDC1 (Fig. 4b). Co-transfection of MDC1 siRNA and siRNA-resistant full-length MDC1 (MDC1^{*}), but not siRNA-resistant MDC1 Δ FHA (MDC1^{*} Δ FHA), restored γ -irradiation-induced apoptosis (Fig. 4c). These results suggest that the interaction of CHK2 and MDC1 is critical for CHK2-mediated DNA damage responses.

We have shown that MDC1 is recruited to activated CHK2 in response to DNA damage. This recruitment is conducted through the interaction between the FHA domain of MDC1 and the phosphorylated Thr 68 site of CHK2. MDC1 is phosphorylated in an ATM- and CHK2-dependent manner and is involved in S-phase checkpoint and radiation-induced apoptosis (Fig. 4d). These results indicate that MDC1 has an important role in CHK2-mediated DNA damage responses. Furthermore, these data demonstrate the importance of the FHA domain–phosphoprotein interaction in the mammalian DNA damage signalling pathways. □

Methods

Plasmids and siRNAs

MDC1 complementary DNA was provided by T. Nagase (Kazusa DNA Research Institute). Wild-type MDC1 or MDC1 Δ FHA was cloned into a pIRES2 vector containing S-protein tag. siRNA-resistant constructs were made by introducing a silent mutation at the MDC1 coding region (70–75; TTGAGG to CTTAGA). The MDC1 FHA and BRCT domains were cloned into the pGEX5.3 vector for bacterial expression of GST fusion proteins. CHK2 constructs have been described previously¹⁵. MDC1 siRNAs were synthesized by Xerogen Inc. The siRNA duplexes were 21 base pairs including a 2-deoxynucleotide overhang. The coding strand of MDC1 siRNA (MDC1 cDNA 58–76) was UCCAGUGAAUCCUUGAGGUdTdT; the control siRNA was UUCAUAAAUCUUGAGGUdTdT.

Antibodies and cell lines

MDC1 antibodies were raised against GST fusion proteins containing amino-terminal residues 1–150 or 151–484 of MDC1. CHK2 antibodies have been previously described¹⁵. Anti- γ H2AX antibodies were generated as previously described¹⁵. Antibodies against p53 and phosphorylated Ser 20 of p53 were purchased from Cell Signaling.

All cells were obtained from American Tissue Culture Collections and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. HCT15 stable cell lines expressing wild-type or mutant CHK2 were generated by neomycin selection.

Immunoprecipitation, immunoblotting and immunostaining

We performed cell lysate preparation, immunoprecipitation, immunoblotting and immunostaining as described¹⁴.

In vitro pull-down assay and peptide competition assay

GST fusion proteins were bound to glutathione-Sepharose overnight at 4 °C. The beads were washed with PBS twice and incubated with cell lysates for 1 h at 4 °C. For peptide competition assay, the indicated concentration of T68 peptide (CETVSTQELYS) and p-T68 peptide (CETVS-pT-QELYS) was added to cell lysates before incubation with beads bound to GST fusion proteins.

siRNA transfection, RDS assay and apoptosis assay

siRNA transfection was performed as described previously²³. Seventy-two hours after initial transfection, cells were γ -irradiated (20 Gy) or left untreated. Cells were then pulsed with [³H]thymidine after the indicated times, and collected 30 min later. For apoptosis assay, cells were irradiated at the indicated doses, and 36 h later fixed and stained with Hoechst. Cells were then scored for apoptosis.

Received 12 December 2002; accepted 27 January 2003; doi:10.1038/nature01447.

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Acknowledgements We thank L. Wang for technical support. We also thank Mayo Protein Core facility for synthesis of peptides. We are grateful to L. Karnitz and S. Kaufmann and members of the Chen and Karnitz laboratories for discussions and ongoing technical support. This work is supported in part by grants from the National Institute of Health, the Prospect Creek Foundation and the Breast Cancer Research Foundation. J.C. is a recipient of a DOD breast cancer career development award.

Competing interests statement The authors declare that they have no competing financial interests.

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MDC1 is a mediator of the mammalian DNA damage checkpoint

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To counteract the continuous exposure of cells to agents that damage DNA, cells have evolved complex regulatory networks called checkpoints to sense DNA damage and coordinate DNA replication, cell-cycle arrest and DNA repair¹. It has recently been shown that the histone H2A variant H2AX specifically controls the recruitment of DNA repair proteins to the sites of DNA damage^{2–4}. Here we identify a novel BRCA1 carboxy-terminal (BRCT) and forkhead-associated (FHA) domain-containing protein, MDC1 (mediator of DNA damage checkpoint protein 1), which works with H2AX to promote recruitment of repair proteins to the sites of DNA breaks and which, in addition, controls damage-induced cell-cycle arrest checkpoints. MDC1 forms foci that co-localize extensively with γ -H2AX foci within minutes after exposure to ionizing radiation. H2AX is required for MDC1 foci formation, and MDC1 forms complexes with phosphorylated H2AX. Furthermore, this interaction is phosphorylation dependent as peptides containing the phosphorylated site on H2AX bind MDC1 in a phosphorylation-dependent manner. We have shown by using small interfering RNA (siRNA) that cells lacking MDC1 are sensitive to ionizing radiation, and that MDC1 controls the formation of damage-induced 53BP1,

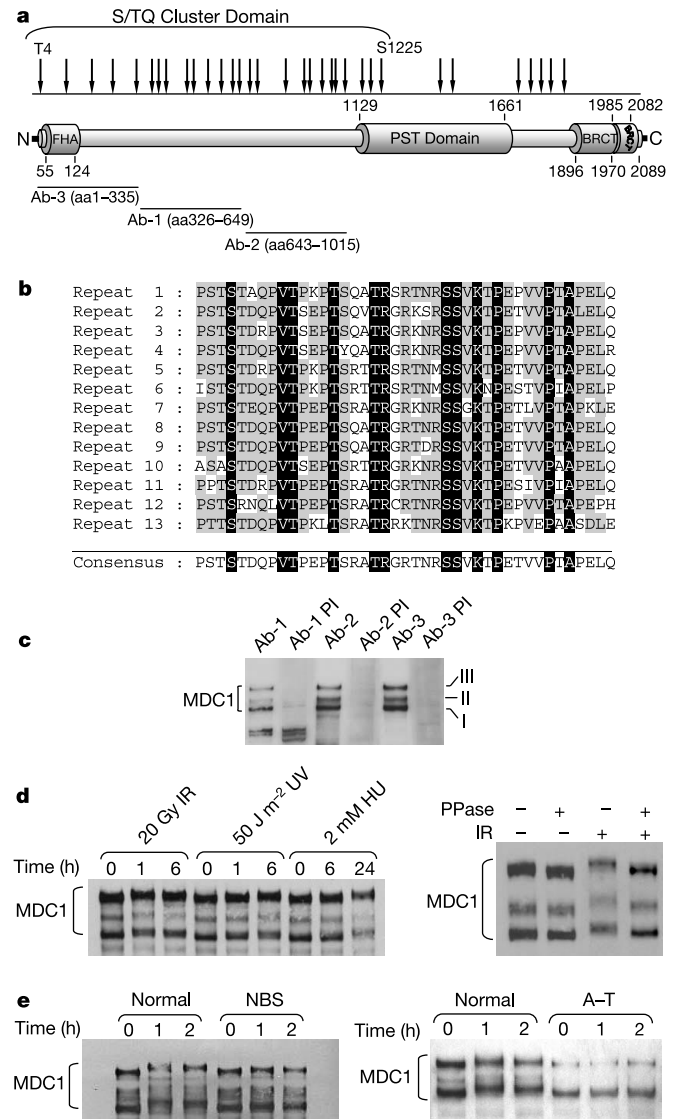


Figure 1 MDC1 is phosphorylated in response to DNA damage and DNA replication stress. **a**, A diagrammatic representation of the MDC1 protein. The amino acids encompassing each domain are indicated. PST indicates proline/serine/threonine repeat domain. The red arrows indicate potential phosphatidylinositol-3-OH kinase-like kinase phosphorylation motifs (SQ/TQ). The fragments of the MDC1 protein used to make anti-MDC1 antibodies (Ab-1, Ab-2, Ab-3) are indicated. **b**, Alignment of the 41-amino-acid repeat sequence that composes the PST domain. Black shaded boxes indicate conserved amino acids and grey boxes indicate similar amino acids. **c**, Recognition of three isoforms (I, II and III) of human MDC1. PI refers to pre-immune serum. **d**, DNA damage-induced phosphorylation of MDC1. Cells were treated with 20 Gy IR, 50 J m⁻² UV or 2 mM HU and harvested at the times indicated. IR-treated cell extracts were also incubated with and without λ protein phosphatase (λ PPase). **e**, MDC1 is phosphorylated in response to IR in an ATM- and Nbs1-dependent manner. Normal, NBS and A-T lymphoblasts were irradiated with 20 Gy of IR and harvested at the times indicated.

BRCA1 and MRN foci, in part by promoting efficient H2AX phosphorylation. In addition, cells lacking MDC1 also fail to activate the intra-S phase and G2/M phase cell-cycle checkpoints properly after exposure to ionizing radiation, which was associated with an inability to regulate Chk1 properly. These results highlight a crucial role for MDC1 in mediating transduction of the DNA damage signal.

Mediators are an emerging class of checkpoint proteins involved in transducing the DNA damage signal. The prototypical mediator

Mediator of DNA Damage Checkpoint Protein 1 Regulates BRCA1 Localization and Phosphorylation in DNA Damage Checkpoint Control*

Received for publication, February 10, 2003,
and in revised form, February 25, 2003
Published, JBC Papers in Press, February 27, 2003,
DOI 10.1074/jbc.C300060200

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BRCA1 is a tumor suppressor involved in DNA repair and damage-induced checkpoint controls. In response to DNA damage, BRCA1 relocates to nuclear foci at the sites of DNA lesions. However, little is known about the regulation of BRCA1 relocation following DNA damage. Here we show that mediator of DNA damage checkpoint protein 1 (MDC1), previously named NFB1 or Kiaa0170, is a proximate mediator of DNA damage responses that regulates BRCA1 function. MDC1 regulates ataxia-telangiectasia-mutated (ATM)-dependent phosphorylation events at the site of DNA damage. Importantly down-regulation of MDC1 abolishes the relocation and hyperphosphorylation of BRCA1 following DNA damage, which coincides with defective G₂/M checkpoint control in response to DNA damage. Taken together these data suggest that MDC1 regulates BRCA1 function in DNA damage checkpoint control.

FHA¹ and BRCT domains are functional modules that are involved in protein-protein interaction (1–3). Many proteins involved in the DNA damage response pathway, such as mammalian BRCA1, 53BP1, Chk2, NBS1, yeast Rad9, and Rad53, contain FHA or BRCT domains. Furthermore mutations within FHA and BRCT domains have been associated with tumorigenesis (4).² These findings suggest important roles of FHA and BRCT domains in DNA damage response pathways.

* This work was supported in part by National Institutes of Health Grants NIH RO1 CA89239 and CA92312 and by the Prospect Creek Foundation and the Breast Cancer Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: FHA, forkhead-associated; BRCT, BRCA1 C-terminal; MDC1, mediator of DNA damage checkpoint protein 1; ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3-related; siRNA, small interfering RNA; H3, histone 3; Gy, gray; IR, ionizing radiation; γH2AX, phospho-H2AX; BARD1, BRCA1-associated RING domain.

² Breast Cancer Information Core at research.nhgri.nih.gov/bic/ on the World Wide Web.

Kiaa0170 or NFB1 (nuclear factor with BRCT domains protein 1) is a nuclear protein that contains both FHA and BRCT domains. Our initial studies on Kiaa0170 and studies from other laboratories have shown that Kiaa0170 forms nuclear foci at the sites of DNA damage and is phosphorylated in an ATM-dependent manner (5–7). Furthermore Kiaa0170 functions as a critical regulator in DNA damage signaling pathways (7, 8).³ Therefore, Kiaa0170 has been renamed as mediator of DNA damage checkpoint protein 1 (MDC1) to better reflect its role in DNA damage checkpoint controls.⁴ In this study, we further explored the role of MDC1 in ATM-dependent DNA damage response pathways.

EXPERIMENTAL PROCEDURES

Plasmids and Small Interfering RNAs (siRNAs)—MDC1 cDNA was kindly provided by Dr. T. Nagase from Kazusa DNA Research Institute (Chiba, Japan). MDC1 siRNAs were synthesized by Xerogen Inc. (Huntsville, AL). The siRNA duplexes were 21 base pairs including a two-deoxynucleotide overhang. The coding strand of MDC1 siRNA1 was UCCAGUGAAUCCUUGAGGUdTdT, and the coding strand of MDC1 siRNA2 was ACAACAUGCAGAGAUUGAAdTdT. The coding strand of BRCA1 siRNA was GGAACUGUCTCCACAAAGdTdT, and the control siRNA was UUCAUAAUUCUUGAGGUdTdT.

Antibodies and Cell Lines—MDC1 antibodies were raised against glutathione S-transferase fusion proteins containing N-terminal residues 1–150 or 151–484 of MDC1. Anti-phospho-H2AX (γH2AX) and anti-BRCA1 antibodies were generated as described previously (8). Anti-p1524BRCA1 antibodies were kindly provided by Dr. KumKum Khanna. Anti-pS317Chk1 antibodies and anti-pATM/ATR were purchased from Cell Signaling. Anti-Chk1 mAb was purchased from Santa Cruz Biotechnology. Anti-phospho-histone 3 antibodies were purchased from Upstate Biotechnology. All cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Immunoprecipitation, Immunoblotting, and Immunostaining—Cell lysate preparation, immunoprecipitation, immunoblotting, and immunostaining were performed as described before (8).

siRNA Transfection—siRNA transfection was performed as described previously (9). Briefly, cells were grown in six-well plate to 30% confluence and immediately before transfection washed with serum-free medium, and 800 μl of serum-free medium were added per well. For each well, 200 nM siRNA was mixed with 5 μl of Oligofectamine (Invitrogen) in 200 μl of serum-free medium. The mixtures were incubated for 20 min at room temperature and then added to cells. Serum was added 4 h later to a final concentration of 10%. 24 h after the initial transfection, a second transfection was performed in the same way as the previous one. 72 h after initial transfection, cells were treated and harvested as indicated.

Phospho-H3 Staining—siRNA-transfected cells were γ irradiated (2 Gy) or left untreated. Cells were then stained with anti-phospho-H3 antibodies, and phospho-H3-positive cells were evaluated by counting or fluorescence-activated cell sorter.

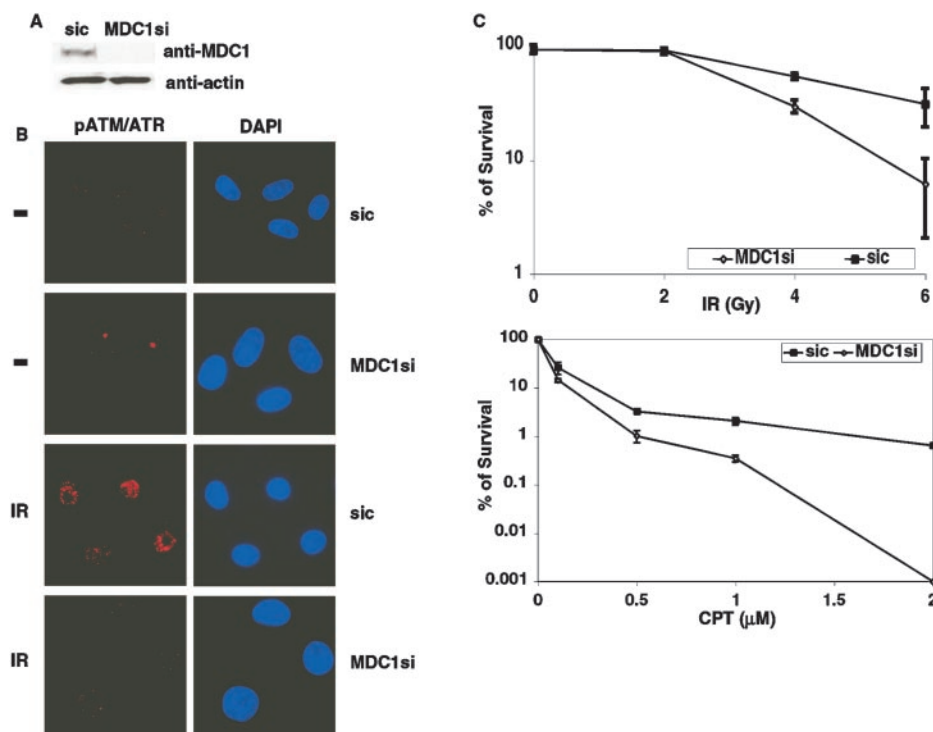
RESULTS AND DISCUSSION

To test the role of MDC1 in ATM-dependent phosphorylation events, we used siRNA technology (9) to down-regulate MDC1 (Fig. 1A) and monitored ATM-dependent phosphorylation events by immunofluorescence staining (Fig. 1B). An antibody raised against phosphopeptide substrates of ATM or ATR has been shown to specifically recognize ATM/ATR-dependent phosphorylation events (10). As shown in Fig. 1B, in cells transfected with control siRNA, ionizing radiation (IR)-induced nuclear foci were present in most irradiated cells, suggesting the accumulation of phosphorylated ATM substrates at the

³ S. Jackson and S. Elledge, personal communication.

⁴ S. J. Elledge and S. P. Jackson, personal communication.

FIG. 1. MDC1 is involved in ATM-dependent phosphorylation events. A and B, HeLa cells were transfected with control siRNA (*sic*) or MDC1 siRNA. After 72 h, cells were lysed and blotted with anti-MDC1 (upper panel) or anti-actin antibodies (lower panel) (A), irradiated (10 Gy) or left untreated, and 16 h later stained with anti-phospho-ATM/ATR substrate antibodies and 4,6-diamidino-2-phenylindole (DAPI) (B). C, HeLa cells were transfected with control siRNA (*sic*) or MDC1 siRNA and treated with IR or camptothecin (CPT). Colony formation was determined 2 weeks later. *MDC1si*, MDC1 siRNA.



sites of DNA breaks. However, down-regulation of MDC1 abolished these IR-induced foci. These results suggest that MDC1 regulates ATM/ATR-dependent phosphorylation events upon DNA damage. Furthermore down-regulation of MDC1 resulted in increased sensitivity to IR and camptothecin (Fig. 1C), implying that MDC1 is required for cell survival following DNA double-stranded breaks. Together these data suggest that MDC1 is involved in ATM-dependent DNA damage response pathways.

In agreement with the role of MDC1 in ATM/ATR-dependent pathways, down-regulation of MDC1 also partially decreased γ H2AX foci staining after IR.⁵ This is different from a previous report (11) that showed intact γ H2AX foci formation in cells transfected with MDC1 siRNA. This discrepancy could be due to the extent of MDC1 down-regulation by different siRNAs used. On the other hand, consistent with the previous report (11), we also observed that down-regulation of H2AX abolished MDC1 foci formation in response to DNA damage.⁵ These findings suggest that H2AX phosphorylation and MDC1 foci formation depend on each other, possibly by forming a positive feedback loop: γ H2AX recruits MDC1 to the sites of DNA damage, which in turn enhances the phosphorylation of H2AX by ATM.

These findings also raise the possibility that γ H2AX and MDC1 cooperate as early signaling molecules to amplify ATM-dependent DNA damage signals. In response to DNA damage, BRCA1 (12) and many other proteins involved in DNA damage responses, such as MRE11/NBS1/Rad50 (13), 53BP1 (8, 14, 15), and Chk2 (16), form nuclear foci. These nuclear foci colocalize with γ H2AX foci, which reside at the sites of DNA damage (17, 18). MDC1 also redistributes to nuclear foci in response to DNA damage (5–7, 11). Similarly MDC1 foci colocalized with BRCA1 foci (Fig. 2A). Furthermore we found that MDC1 co-immunoprecipitated with BRCA1. This interaction is constitutive, occurring independently of DNA damage (Fig. 2B). Given that BRCA1 forms a stable heterodimeric complex with BARD1 *in*

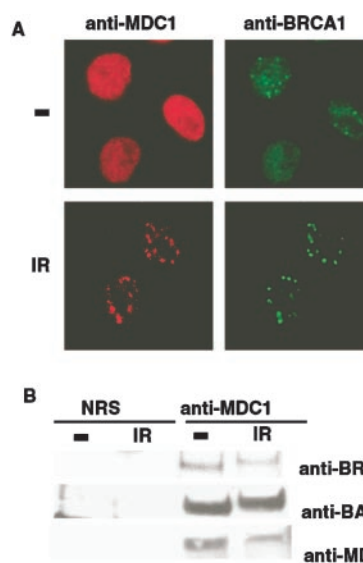


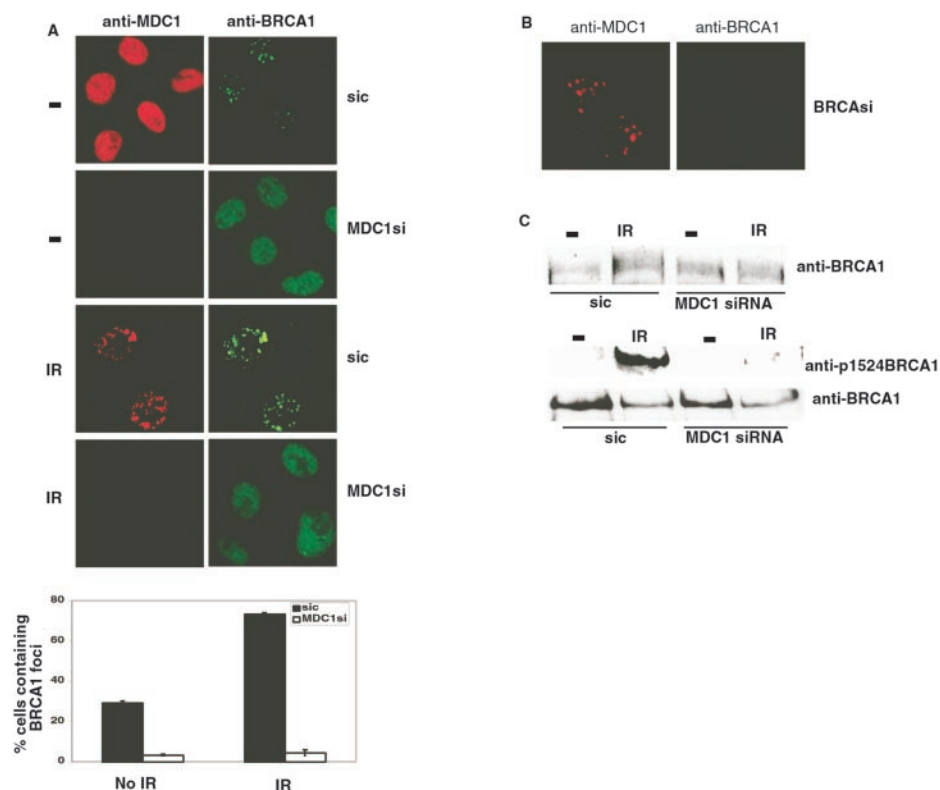
FIG. 2. MDC1 interacts with BRCA1-BARD1. A, HeLa cells were irradiated (10 Gy) or left untreated and 16 h later stained with anti-BRCA1 and anti-MDC1 antibodies as indicated. B, K562 cells were irradiated or left untreated, and MDC1 was then immunoprecipitated with normal rabbit serum (NRS) or anti-MDC1 antibodies. The immunoprecipitates were blotted with anti-BRCA1, anti-BARD1, and anti-MDC1 antibodies.

vivo (19), we further determined whether MDC1 could also associate with BARD1. As shown in Fig. 2B, similar to BRCA1, BARD1 also associates with MDC1. These results suggest that MDC1 interacts with the BRCA1-BARD1 complex and may be involved in a BRCA1-mediated genome maintenance function.

The interaction between MDC1 and BRCA1 led us to investigate the relationship between MDC1 and BRCA1. We used siRNA technology to down-regulate either BRCA1 or MDC1. Transfection of HeLa cells with BRCA1 siRNA or MDC1 siRNA resulted in the depletion of BRCA1 and MDC1, respectively (Figs. 3, A and B, and 4A). As shown in Fig. 3A, depletion of MDC1 by siRNA transfection led to a complete loss of BRCA1

⁵ Z. Lou, C. C. S. Chini, K. Minter-Dykhouse, and J. Chen, unpublished observation.

FIG. 3. MDC1 regulates BRCA1 foci formation and phosphorylation. A, HeLa cells were transfected with control siRNA or MDC1 siRNA, irradiated (10 Gy) or left untreated, and then stained with anti-BRCA1 and anti-MDC1 antibodies. Quantification of cells containing the indicated foci is shown in the lower panel. B, HeLa cells were transfected with control siRNA or BRCA1 siRNA. After 72 h, cells were irradiated (10 Gy) and 16 h later stained with anti-MDC1 and anti-BRCA1 antibodies as indicated. C, HeLa cells were transfected with control siRNA or MDC1 siRNA, irradiated (10 Gy) or left untreated, and then lysed and blotted with the indicated antibodies. *sic*, control siRNA; *MDC1si*, MDC1 siRNA.



foci in response to DNA damage (from 80% of cells containing BRCA1 foci in control samples to 4% in the MDC1 siRNA-transfected sample) (Fig. 3A). Only cells that retained MDC1 expression still formed BRCA1 foci (data not shown). Conversely, although transfection of HeLa cells with BRCA1 siRNA resulted in a loss of BRCA1 in 90% of the transfected cells (Fig. 3B and data not shown), the relocalization of MDC1 to nuclear foci following DNA damage was not affected by the depletion of BRCA1 (Fig. 3B). Furthermore the disappearance of BRCA1 foci in MDC1 siRNA-transfected cells is not due to a change in kinetics of BRCA1 foci formation since we could not detect BRCA1 foci either 6 h (data not shown) or 16 h after DNA damage (Fig. 3A). In unirradiated control cells, there are a subset of cells that contain BRCA1 foci (30%), which are the previously reported S-phase BRCA1 foci (12). Interestingly transfection of MDC1 siRNA in these cells led to a dramatic decrease of cells containing these BRCA1 S-phase foci (2% of cells containing BRCA1 foci) (Fig. 3A), suggesting that BRCA1 S-phase foci formation may also require MDC1. Transfection with a control siRNA, which was generated by introducing point mutations into MDC1 siRNA, had no effect on BRCA1 foci formation (Fig. 3A). Furthermore transfection with a second, different MDC1 siRNA (siRNA2) also abolished the localization of BRCA1 to nuclear foci (data not shown). Taken together these data strongly suggest that MDC1 is required for the recruitment of BRCA1 to the sites of DNA damage.

Using siRNA technology, we also found that down-regulation of H2AX significantly decreased IR-induced BRCA1 foci (data not shown). This is in agreement with early studies using H2AX-deficient mouse cells (20, 21). However, it is unlikely that MDC1 regulates BRCA1 foci formation solely through influencing γ H2AX since MDC1 siRNA only partially reduced γ H2AX foci (data not shown) but completely abolished BRCA1 foci in response to IR (Fig. 3A). In addition, while MDC1 regulated BRCA1 S-phase foci formation (Fig. 3A), H2AX does not seem to be required for the formation of BRCA1 S-phase foci (data not shown and see Ref. 20). Finally, while H2AX is

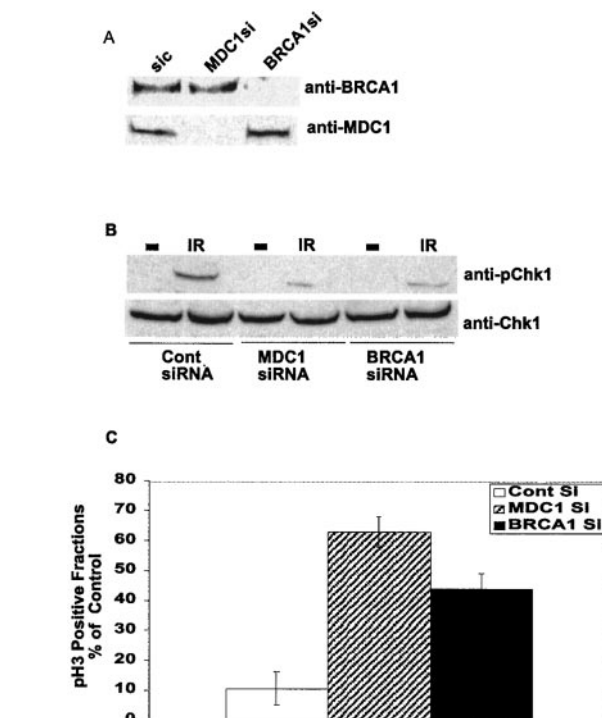


FIG. 4. MDC1 regulates Chk1 activation and G_2/M checkpoint in response to DNA damage. A and B, HeLa cells transfected with control siRNA, MDC1 siRNA, or BRCA1 siRNA were irradiated or left untreated, and cell lysates were blotted with the indicated antibodies. C, defective G_2/M checkpoint in cells transfected with MDC1 siRNA. HeLa cells transfected with control siRNA, MDC1 siRNA, or BRCA1 siRNA were irradiated (2 Gy) or left untreated. 1 h later cells were fixed and stained with anti-phospho-H3 antibodies, and phospho-H3-positive fractions were determined. The results were expressed as the percentage of the phospho-H3-positive fraction in irradiated cells compared with that in unirradiated control cells. *sic*, control siRNA; *MDC1si*, MDC1 siRNA; *BRCA1si*, BRCA1 siRNA; *Cont*, control; *Si*, siRNA; *pH3*, phospho-H3.

dispensable for DNA damage-induced hyperphosphorylation of BRCA1 (data not shown), MDC1 is required for BRCA1 phosphorylation following DNA damage (see below).

BRCA1 is phosphorylated by ATM at multiple residues including Ser-1387, Ser-1423, Ser-1457, and Ser-1524 after irradiation (22, 23). To further investigate the role of MDC1 in the regulation of BRCA1, we examined the phosphorylation of BRCA1 following DNA damage in MDC1-deficient cells. As shown in Fig. 3C, transfection with MDC1 siRNA resulted in defective hyperphosphorylation of BRCA1 following DNA damage as judged by its mobility shift by SDS-PAGE or by blotting with antibodies against phosphoserine 1524 of BRCA1. However, BRCA1 phosphorylation is normal in cells transfected with H2AX siRNA (data not shown). It is worth mentioning that the defective BRCA1 phosphorylation in MDC1 siRNA-transfected cells is only observed with low doses of irradiation (10 Gy or lower). When higher doses of irradiation are used (30 Gy), phosphorylation of BRCA1 observed in MDC1 siRNA-transfected cells is similar to that in control cells (data not shown), suggesting the possibility that an alternative pathway for BRCA1 phosphorylation is activated at higher doses. Recently 53BP1 has also been reported to regulate BRCA1 foci formation and phosphorylation (24). It is possible that MDC1 and 53BP1 might work together in regulating BRCA1 following DNA damage.

The defective BRCA1 localization and phosphorylation in MDC1 siRNA-transfected cells suggests that MDC1 might regulate BRCA1-mediated functions. It is well established that BRCA1 is involved in the maintenance of genome stability. BRCA1 participates in a number of activities following DNA damage including DNA repair (25), Chk1 activation (26), S-phase and G₂/M checkpoint control (27, 28), and cell survival (22). This prompted us to investigate how MDC1 affects BRCA1-mediated downstream events. Using MDC1 siRNA or BRCA1 siRNA, which specifically down-regulated MDC1 and BRCA1, respectively (Fig. 4A), we first examined the Chk1 activation following DNA damage. In response to DNA damage, Chk1 is phosphorylated at Ser-317 and Ser-345, and the phosphorylation of these sites is required for Chk1 activation (29). Down-regulation of either BRCA1 or MDC1 resulted in decreased Chk1 phosphorylation following DNA damage (Fig. 4B). BRCA1 has recently been shown to regulate the G₂/M checkpoint, probably through its regulation of Chk1 activation (26). Thus, we next examined the G₂/M checkpoint control in cells depleted of MDC1. As shown in Fig. 4C, HeLa cells transfected with control siRNA showed a marked decrease in mitotic fraction in response to γ irradiation. However, similar to cells transfected with BRCA1 siRNA, cells transfected with MDC1 siRNA showed significantly greater mitotic fraction after γ irradiation, suggesting a G₂/M checkpoint defect in cells lacking MDC1 or BRCA1. Taken together these results suggest that MDC1 is involved in the G₂/M checkpoint, probably through regulating the BRCA1-Chk1 pathway.

Emerging evidence suggests that MDC1 is an important mediator of DNA damage responses. In addition to its role in BRCA1 foci formation and phosphorylation, MDC1 also interacts with activated Chk2 and regulates S-phase checkpoint, p53 stabilization, and radiation-induced apoptosis (7). A recent report also links MDC1 to the Chk2 pathway (11). Furthermore we and others have shown that MDC1 regulates NBS1 foci formation.^{3,5} These findings suggest that MDC1 regulates multiple DNA damage signaling pathways. MDC1 contains several protein-protein interaction domains including the FHA do-

main, BRCT domain, and internal repeated sequences. It is plausible that MDC1 acts as an adaptor protein to recruit downstream signaling molecules to the sites of DNA damage in a role similar to that of Grb2 or Shc in receptor tyrosine kinase-mediated signaling pathways.

In summary, we have shown that MDC1 regulates BRCA1 foci formation, phosphorylation, and G₂/M checkpoint control in response to DNA damage. These results establish a critical role of MDC1 in the regulation of DNA damage checkpoint. Given that dysregulation of DNA damage checkpoints (e.g. mutations of p53 and BRCA1) frequently leads to tumorigenesis, one may wonder whether MDC1 functions as a tumor suppressor. Future genetic studies of MDC1 will be conducted to test this possibility.

Acknowledgments—We thank Dr. T. Nagase from Kazusa DNA Research Institute for providing MDC1 (Kiaa0170) cDNA and Dr. KumKum Khanna for providing anti-phospho-BRCA1 antibodies. We also thank the Mayo Protein Core facility for synthesis of peptides. We are extremely grateful to Drs. Larry Karnitz and Scott Kaufmann and members of the Chen and Karnitz laboratories for helpful discussions and ongoing technical support.

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MDC1 Maintains Genomic Stability by Participating in the Amplification of ATM-Dependent DNA Damage Signals

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Summary

MDC1 functions in checkpoint activation and DNA repair following DNA damage. To address the physiological role of MDC1, we disrupted the MDC1 gene in mice. MDC1^{−/−} mice recapitulated many phenotypes of H2AX^{−/−} mice, including growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects, and radiation sensitivity. At the molecular level, H2AX, MDC1, and ATM form a positive feedback loop, with MDC1 directly mediating the interaction between H2AX and ATM. MDC1 binds phosphorylated H2AX through its BRCT domain and ATM through its FHA domain. Through these interactions, MDC1 accumulates activated ATM flanking the sites of DNA damage, facilitating further ATM-dependent phosphorylation of H2AX and the amplification of DNA damage signals. In the absence of MDC1, many downstream ATM signaling events are defective. These results suggest that MDC1, as a signal amplifier of the ATM pathway, is vital in controlling proper DNA damage response and maintaining genomic stability.

Introduction

The DNA damage-response pathway is essential for the maintenance of genomic stability (Rouse and Jackson, 2002; Zhou and Elledge, 2000). It is a network of signaling pathways that responds to DNA damage and activates cell cycle checkpoints, DNA repair, and apoptosis. Dysfunction of the DNA damage-response pathway re-

sults in genomic instability, which is a driving force of tumorigenesis (Hahn and Weinberg, 2002).

In response to DNA damage, many proteins involved in the DNA damage signaling pathway, including BRCA1, MRE11/NBS1/Rad50, and 53BP1, quickly relocalize to nuclear foci (Paull et al., 2000; Rappold et al., 2001; Schultz et al., 2000; Scully et al., 1997). These foci colocalize with phosphorylated H2AX (γH2AX) foci, which are well established as the earliest detectable markers for double-strand breaks (DSBs) (Rogakou et al., 1998). Interestingly, phosphorylated H2AX does not just localize at the sites of DNA damage but quickly spreads to the surrounding megabase regions (Rogakou et al., 1999). It is likely that this buildup of phosphorylated H2AX is the signal that leads to the retention of DNA damage-response factors, such as 53BP1 and NBS1, at the sites of DNA breaks (Bassing et al., 2002; Celeste et al., 2002, 2003). γH2AX may serve as a docking site for these DNA damage/repair proteins (Celeste et al., 2003). Alternatively, γH2AX may modulate chromatin structure and thus indirectly facilitate the accumulation of checkpoint proteins (Fernandez-Capetillo et al., 2003). However, it remains unclear what assists in the spreading of H2AX phosphorylation to the megabase regions surrounding the DNA damage sites.

Recent studies suggest that mediator of DNA damage checkpoint protein 1 (MDC1) regulates many aspects of the DNA damage-response pathway, such as intra-S phase checkpoint, G2/M checkpoint and radiation-induced apoptosis (Goldberg et al., 2003; Lou et al., 2003a, 2003b; Peng and Chen, 2003; Shang et al., 2002; Stewart et al., 2003; Xu and Stern, 2002). MDC1 also regulates foci formation of NBS1/MRE11/Rad50, 53BP1, and BRCA1 (Goldberg et al., 2003; Lou et al., 2003a; Stewart et al., 2003). Since MDC1 contains protein-protein interaction domains, such as the FHA and the BRCT domain, it is believed that MDC1 functions as an adaptor protein. However, the mechanism by which MDC1 regulates so many aspects of the DNA damage-response pathway is not fully understood. In addition, the physiological function of MDC1 in a living organism has not been investigated. With these goals in mind, we generated MDC1 knockout mice and studied the physiological function of MDC1 in DNA damage response.

Results

To explore the endogenous function of MDC1, we obtained mouse ES cells from Omnibank (Zambrowicz et al., 1998). The MDC1 gene was disrupted by the gene-trapping method, with the retroviral vector VICTR48 inserted between exon 1 and exon 2 of the mouse MDC1 gene (Figure 1A). These ES cells were then used to create chimeric mice from which MDC1^{−/−} mice were generated. MDC1^{−/−} mice were born at the expected frequency (data not shown). RT-PCR with primers flanking the insertion site revealed the disruption of MDC1 transcript (data not shown). The loss of MDC1 expression was further confirmed by immunoblotting

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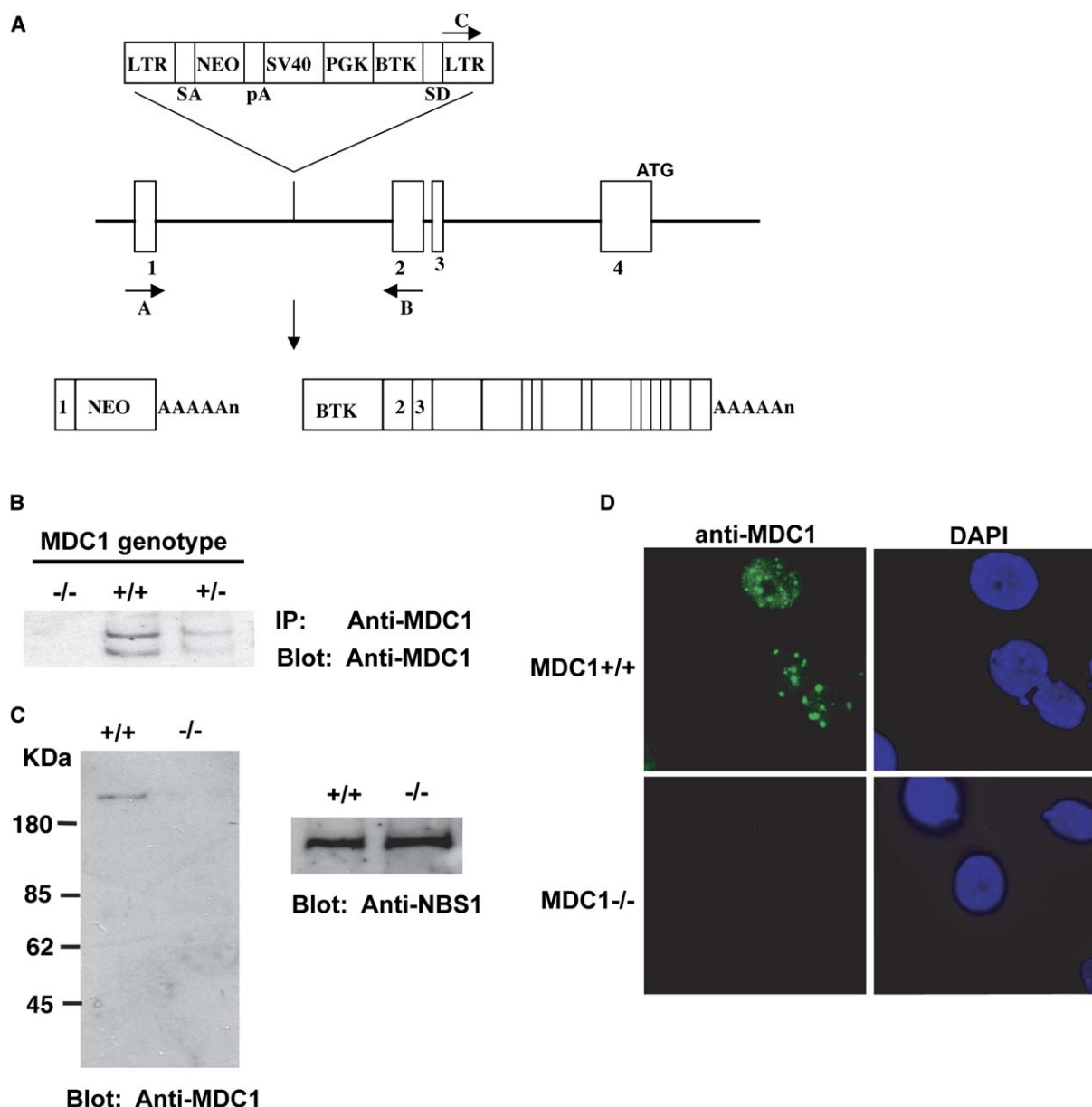


Figure 1. Generation of MDC1-Deficient Mice

(A) Schematic diagram of the disruption of mouse MDC1 locus. Arrows represent the position and orientation of PCR primers used for genotyping. Splicing of the neomycin gene and flanking DNA produces a transcript that potentially disrupts the proper splicing of exons 1 and 2 of the MDC1 gene (lower panel). LTR, long-terminal repeat; NEO, neomycin resistance gene; pA, poly-A; PGK, phosphoglycerate kinase-1; BTK, Bruton's tyrosine kinase; SA and SD, splice acceptor and donor, respectively.

(B) Extracts from mouse testes were immunoprecipitated and blotted using antibodies against the N terminus of MDC1.

(C) (Left panel) Whole-cell extracts from MDC1^{+/+} and MDC1^{-/-} MEFs were blotted with antibodies against the C terminus of MDC1. (Right panel) Immunoblot of NBS1 was included as loading control.

(D) Immunostaining of irradiated (1 Gy) MDC1^{+/+} and MDC1^{-/-} MEFs were performed using antibodies against the central region of MDC1.

using anti-MDC1 antibodies recognizing the N terminus or the C terminus of MDC1 (Figures 1B and 1C). No full-length or truncated MDC1 was detected in MDC1^{-/-} samples. In addition, no signal was detected by immunofluorescence staining in MDC1^{-/-} mouse embryonic fibroblasts (MEFs), using another anti-MDC1 antibody that specifically recognizes the central region of MDC1 (Figure 1D).

The MDC1^{-/-} mice were growth retarded. The average weight of 5-month-old MDC1^{-/-} mice was 80% of that of wild-type mice (Figure 2A). The defective growth of MDC1^{-/-} mice correlated with the reduced proliferation of MDC1^{-/-} cells in vitro (Figure 2B). MEFs from MDC1^{-/-} mice grew poorly in vitro due to a decrease in actively dividing cells, judged by BrdU staining (data not shown). This was accompanied by the accumulation

of giant nondividing cells, possibly due to premature senescence (data not shown).

Multiple attempts to breed male MDC1^{-/-} mice with female wild-type mice failed to yield any pregnancies, suggesting that male MDC1^{-/-} mice are infertile. Although female MDC1^{-/-} mice are fertile, their litter sizes were smaller than those of wild-type mice. The infertility of male MDC1^{-/-} mice was associated with defective spermatogenesis, since we observed that testes from MDC1^{-/-} mice were only one-fourth the size of those of wild-type mice at the same age (Figure 2C). Consistent with the reduced testicular size, the diameter of the seminiferous tubules in MDC1^{-/-} mice was smaller than those in wild-type mice (Figure 2D). Although spermatocytes of all developmental stages were identified in seminiferous tubules from wild-type mice, far fewer spermatocytes were found in seminiferous tubules from MDC1^{-/-} mice. The male infertility phenotype has also been reported in ATM^{-/-} and H2AX^{-/-} animals, albeit due to distinctive defects during meiosis (Barlow et al., 1996; Celeste et al., 2002; Elson et al., 1996; Xu et al., 1996). Exactly how MDC1 regulates spermatogenesis requires further investigation.

Many factors involved in DSB repair, such as DNA-PKcs/Ku, ATM, and H2AX, have been shown to regulate immunological processes. To assess the role of MDC1 in lymphocyte maturation, we examined bone marrow from MDC1^{+/+} and MDC1^{-/-} mice and found no significant difference in the percentage of pro-B (B220+CD43^{lo}) and pre-B (B220+CD43^{lo}) cells (data not shown), consistent with no major developmental block at the stage when V(D)J recombination occurs. Compared to wild-type mice, the thymus and spleen derived from MDC1^{-/-} mice are smaller; however, the absolute number of splenic B and T lymphocytes is only modestly reduced ($1.63 \pm 0.42 \times 10^7$ versus $1.16 \pm 0.38 \times 10^7$ for B lymphocytes; $5.30 \pm 1.8 \times 10^7$ versus $4 \pm 1.3 \times 10^7$ for T lymphocytes), suggesting that peripheral B and T cell development is overall normal in the absence of MDC1.

Since class switch recombination (CSR) also requires the regulated cleavage and repair of DNA, we sought to examine whether IgH class switching is affected in MDC1^{-/-} mice. Serum IgH concentrations were similar in MDC1^{-/-} mice as compared to wild-type littermate controls (data not shown). Purified splenic B cells were stimulated *in vitro* for class switching and analyzed for secretion and surface expression of specific immunoglobulin isotypes. Concentrations of IgM are comparable in supernatants from MDC1^{-/-} and wild-type B cell cultures, indicating normal activation and proliferation of B cells after *in vitro* stimulation (data not shown). Following anti-CD40/IL4 treatment, surface expression of IgG1 was reduced between 25% and 50% in MDC1^{-/-} cultured B cells (Figure 2E), whereas expression of IgG2b and IgG3 following LPS stimulation was decreased when compared to wild-type, but this did not approach statistical significance (Figure 2E). Supernatants from these B cell cultures were analyzed for secreted Ig, and only a modest decrease in IgG1 concentration was observed, with no significant difference seen for IgG2b or IgG3 (data not shown). Further evidence supporting a modest class switch defect was obtained from hybridomas generated from anti-CD40/IL4-stimulated B cells with half the number of IgG1-se-

creting clones recovered from MDC1^{-/-} cultures as compared to wild-type (Figure 2F). Taken together, these data indicate that MDC1 deficiency results in a mild defect in Ig class switching, similar to that seen for ATM^{-/-} but not as severe as H2AX or 53BP1 deficiency.

One of the hallmarks of defective DNA damage responses is increased radiation sensitivity. To test if the loss of MDC1 expression renders mice hypersensitive to ionizing radiation (IR), we irradiated MDC1^{+/+}, MDC1^{+/-} and MDC1^{-/-} mice. As shown in Figure 3A, all MDC1^{-/-} mice died within 16 days after 7 Gy of irradiation, while 80% of MDC1^{+/+} and MDC1^{+/-} mice were still alive 2 months after irradiation. On the cellular level, MDC1^{-/-} MEFs were also hypersensitive to low-dose irradiation (Figure 3B). To characterize cytologically how MDC1^{+/+} and MDC1^{-/-} cells respond to DNA damage, we examined metaphase spreads of MDC1^{+/+} and MDC1^{-/-} MEFs (passage 1) 3 hr after IR. MDC1^{-/-} cells displayed far more DNA breaks than did MDC1^{+/+} cells (Figure 3C). MDC1^{+/+} cells efficiently repaired the majority of DSBs, with 22% of the cells having more than one break (Figure 3D). In contrast, MDC1^{-/-} cells show severe defects in DSB repair, with 92% of the cells having more than one break. These results suggest that MDC1 functions in DSB repair.

Failure to repair DNA damage is linked to genomic instability. The observed defects in meiosis, class switching, and DSB repair in MDC1^{-/-} mice suggest that MDC1 plays an important role in maintaining genomic stability. We used metaphase spreads of activated T cells to examine genomic stability of MDC1^{+/+} and MDC1^{-/-} cells in the absence of exogenous DNA damaging agents. Few MDC1^{+/+} cells (3.5%) displayed spontaneous chromosome aberrations (Figures 3E and 3F). In contrast, significantly more MDC1^{-/-} cells (17.5%) show spontaneous chromosome aberrations, including chromosome breaks, chromatid breaks, fragments, and dicentric chromosomes (Figures 3E and 3F). These results confirm that MDC1 plays a critical role in maintaining genomic stability.

The phenotypes we observed from MDC1^{-/-} mice are very similar to those observed in H2AX^{-/-} mice. On the molecular level, both H2AX and MDC1 are required for the accumulation of multiple DNA damage factors flanking DNA breaks, such as the M/R/N complex, BRCA1, and 53BP1. However, exactly how MDC1 regulates the accumulation of so many DNA damage factors was unclear. We hypothesized that MDC1 could promote H2AX phosphorylation, thus directly or indirectly affecting the accumulation of DNA damage factors.

To test this hypothesis, we performed kinetic studies of H2AX phosphorylation. Because of the poor growth of primary MEFs, all the following experiments were performed using spontaneously immortalized MEFs. In MDC1^{+/+} cells, H2AX was rapidly phosphorylated, and γ H2AX foci could be observed within minutes of IR in wild-type cells (see Figure S1 in the Supplemental Data available with this article online). The number of foci corresponded to predicated DSBs (about 30 DSBs per Gy). Further time course analysis revealed that the γ H2AX foci became bigger and brighter 10 and 30 min after IR. The number of bright γ H2AX foci decreased 1 hr after IR, probably due to the repair of DNA breaks. On the other hand, we found that, although γ H2AX

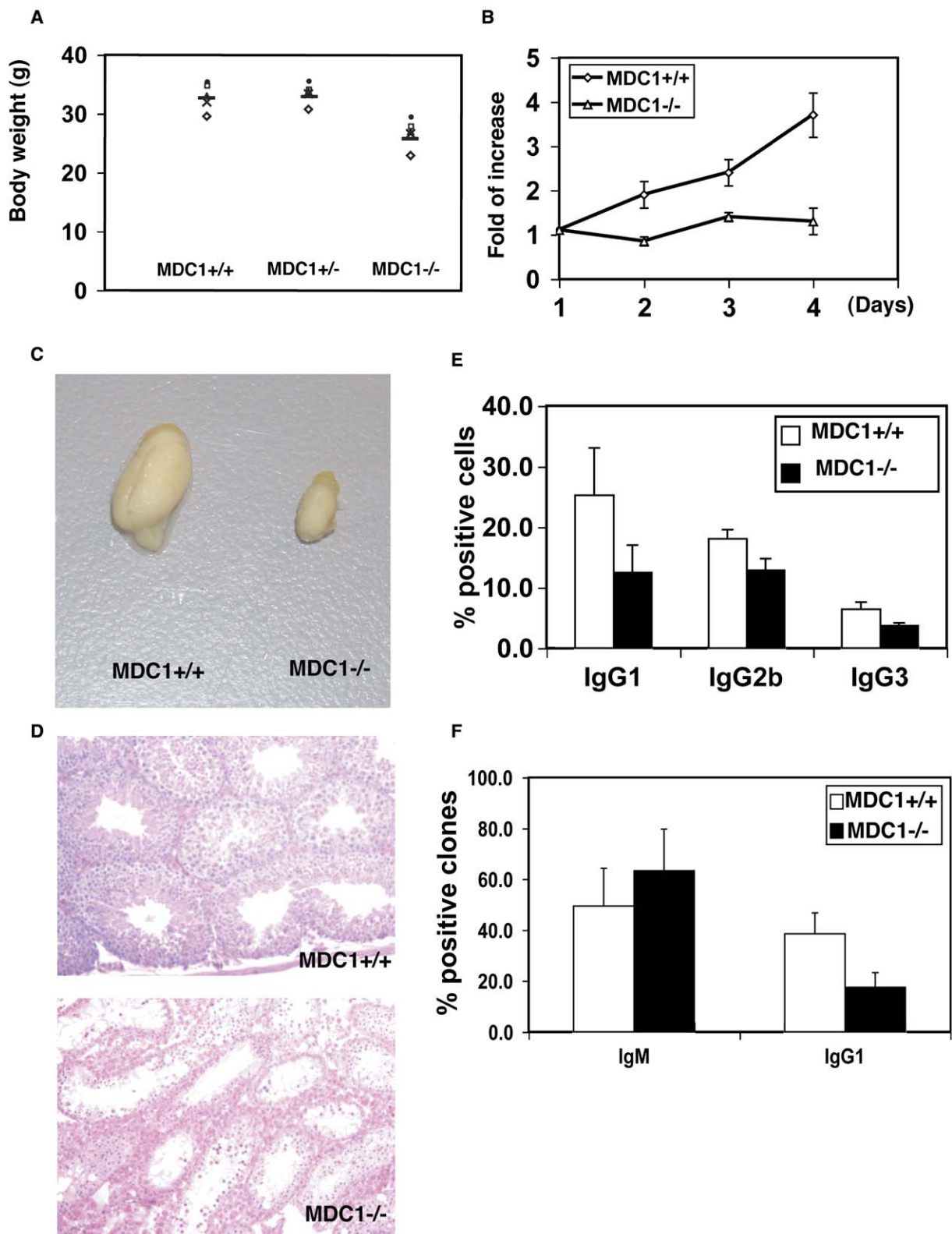


Figure 2. MDC1^{-/-} Mice Show Growth Retardation and Male Infertility

(A) Body weights of male MDC1^{+/+}, MDC1^{+/-} and MDC1^{-/-} mice were evaluated at 5 months of age.

(B) MDC1^{-/-} MEFs showed reduced proliferation in vitro. MDC1^{+/+} and MDC1^{-/-} MEFs were cultured in 6-well plates at a density of 10⁵ per well. Each day, one set of cells was trypsinized and counted. The Y axis represents average folds of increase in cell numbers. The error bar represents the standard error of three independent experiments.

(C) Comparison of testis size in 2-month-old MDC1^{+/+} and MDC1^{-/-} mice.

rapidly formed foci following IR in MDC1^{-/-} cells, the intensity of γ H2AX foci was significantly reduced than those in wild-type cells (Figure S1). The number of foci observed did not correspond to predicated DSBs. This is probably due to weaker γ H2AX signals that were below the threshold of detection. In addition, there were no obvious changes in the intensity or numbers of γ H2AX foci after initial γ H2AX signals. To further examine the kinetics of H2AX phosphorylation following IR, we extracted histones and performed Western blot analysis using anti- γ H2AX antibodies. In wild-type cells, H2AX phosphorylation increased following IR and peaked at 30 min post IR (Figure 4A). Although H2AX is rapidly phosphorylated following IR in MDC1^{-/-} cells, H2AX phosphorylation was weaker and failed to propagate in response to DNA damage. These results suggest an important role of MDC1 in promoting or amplifying H2AX phosphorylation.

As a mediator protein, MDC1 may promote H2AX phosphorylation by mediating the interaction between an upstream kinase and H2AX. ATM has been shown to be the major kinase that phosphorylates H2AX (Burma et al., 2001; Fernandez-Capetillo et al., 2002), although DNA-PK may also function redundantly with ATM in phosphorylating H2AX under certain growth conditions (Stiff et al., 2004). Our studies using siRNA to downregulate ATM, ATR, DNA-PK clearly demonstrated the important role of ATM in H2AX phosphorylation (Figure S2).

ATM is activated by autophosphorylation at Ser1981 following DNA damage. As a result, the inactive ATM dimer disassociates and becomes an active monomer (Bakkenist and Kastan, 2003). Activated ATM then accumulates at the sites of DNA breaks (Bakkenist and Kastan, 2003). It is possible that MDC1 regulates either ATM activation or ATM accumulation near the sites of DSBs. We repeatedly observed comparable ATM autophosphorylation in MDC1^{+/+} and MDC1^{-/-} cells after treating these cells with different doses of radiation (Figure 4B). These results suggest that MDC1 does not play an important role in ATM activation. To test if MDC1 is required for the accumulation of activated ATM around the sites of DNA damage, we performed immunofluorescence-staining studies using anti-phospho-ATM antibodies. Activated ATM accumulates near DSBs (Bakkenist and Kastan, 2003), as demonstrated by the foci formation detected by anti-phospho-S1981 ATM antibodies (Figure 4C). Interestingly, activated ATM completely failed to accumulate near DSBs in MDC1^{-/-} cells, even though phospho-ATM immunostaining signals were increased following DNA damage. Similar results were observed when MDC1 siRNA was used to downregulate MDC1 in HeLa cells (Figure S4). These results indicate that MDC1 regulates the accumulation of ATM near DSBs. To confirm the role of MDC1 in DNA damage-induced ATM accumulation, we per-

formed a chromatin fractionation assay. In MDC1^{+/+} cells, ATM became enriched in chromatin-rich insoluble fractions (fraction III) following DNA damage (Figure 4D). However, no enrichment of ATM in chromatin-rich insoluble fractions was observed in MDC1^{-/-} cells (Figure 4D). These results suggest that MDC1 regulates the accumulation of ATM at the sites of DNA damage.

The role of MDC1 in ATM accumulation led us to hypothesize that MDC1 might regulate the access of ATM to H2AX, therefore promoting the expansion of H2AX phosphorylation following DNA damage. ATM bound H2AX beads in a phosphorylation-dependent manner in the peptide pull-down assay (Figure 4E). The binding of ATM to γ H2AX beads does not require full-length NBS1 (Figure S5). Interestingly, in the absence of MDC1, ATM fails to bind γ H2AX beads (Figure 4F). These results are consistent with our hypothesis that MDC1 mediates the γ H2AX-ATM interaction.

MDC1 has previously been shown to bind both ATM and γ H2AX (Lukas et al., 2004; Stewart et al., 2003; Xu and Stern, 2003). However, it is not clear if these interactions are direct. To test if MDC1 directly mediates the interaction between γ H2AX and ATM, we did in vitro binding assays using purified ATM and MDC1. As shown in Figure 4G, H2AX peptide interacts with purified MDC1 in a phosphorylation-dependent manner in vitro. Significantly, while ATM did not interact with γ H2AX, addition of purified MDC1 promoted the interaction between ATM and γ H2AX (Figure 4G). These results demonstrate that MDC1 directly mediates the interaction between ATM and γ H2AX.

We next asked mechanistically how MDC1 interacts with γ H2AX. MDC1 contains two domains that recognize phospho-S/T motifs, the FHA domain and the BRCT domain (Durocher et al., 1999; Manke et al., 2003; Yu et al., 2003). We generated GST fusion proteins containing either the FHA or the BRCT domain of MDC1. The GST-FHA or the GST-BRCT domain of MDC1 was then incubated with S139-phosphorylated (γ H2AX) or nonphosphorylated H2AX peptide. As shown in Figure 5A, the BRCT domain of MDC1 binds H2AX in a phosphorylation-dependent manner. Interestingly, the sequence surrounding pS139 (pSXXY) of H2AX fits perfectly with the predicted binding site of the MDC1 BRCT domain (Rodriguez et al., 2003). Deletion of the BRCT domain abolished the γ H2AX-MDC1 interaction (Figure 5B) and the ability of MDC1 to form foci at the sites of DNA damage (Figure 5F). These results suggest that MDC1 directly interacts with phosphorylated H2AX through its BRCT domain and that this interaction is required for MDC1 focus localization following DNA damage.

To characterize the ATM-MDC1 interaction, we again used GST, GST-FHA, and GST-BRCT domain of MDC1 to perform pull-down assays. As shown in Figure 5C, the FHA domain of MDC1 specifically interacted with

(D) Hematoxylin and eosin staining of sections of seminiferous tubules of 2-month-old MDC1^{+/+} and MDC1^{-/-} mice.

(E) Splenic B cells from MDC1^{-/-} animals and littermate controls were isolated and stimulated in culture to undergo class switching either with lipopolysaccharide (LPS, switching to C γ 2b and C γ 3) or with anti-CD40 antibody and interleukin-4 (anti-CD40/IL4, switching to C γ 1 and C ϵ). Surface immunoglobulin expression was then assessed on day 4.5 by flow cytometry. The error bar represents the standard error of results from six littermates.

(F) Hybridomas were obtained from purified splenic B cells stimulated for 4 days in culture with anti-CD40/IL4 and analyzed for IgH isotype secretion by ELISA. A total of 531 independent hybridomas from three wild-type mice and of 375 independent hybridomas from three MDC1^{-/-} mice were analyzed. The error bar represents the standard error of results from three littermates.

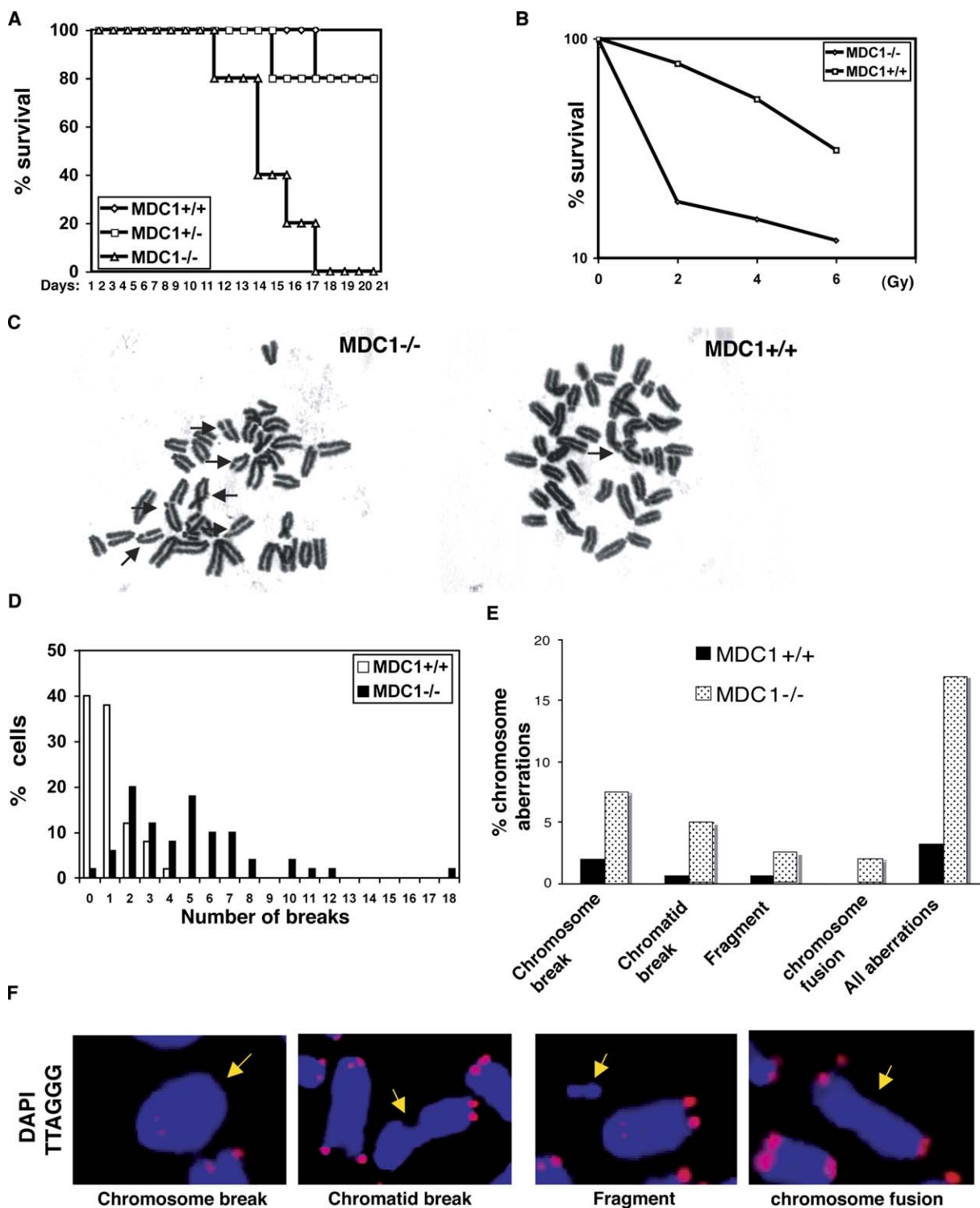


Figure 3. MDC1^{-/-} Mice Have Increased Radiation Sensitivity and Defective DNA Repair

(A) Survival curve of MDC1^{+/+}, MDC1^{+/-}, and MDC1^{-/-} littermates after whole-body exposure to 7 Gy of IR.

(B) Increased radiation sensitivity was observed in passage 1 MDC1^{-/-} MEFs. The Y axis represents the percentage of surviving cells relative to unirradiated control cells of the same genotypes.

(C and D) MDC1^{-/-} cells show defective DNA repair. (C) Shown are examples of metaphases prepared from MDC1^{+/+} and MDC1^{-/-} passage 1 MEFs 3 hr after treatment of 1 Gy of IR. Arrows, DNA breaks. (D) Chromosome aberrations observed in MDC1^{+/+} and MDC1^{-/-} passage 1 MEFs after treatment with 1 Gy of IR were quantified. Metaphase spreads (100) were evaluated for each genotype.

(E and F) MDC1^{-/-} cells show increased genomic instability. (E) Numbers and spectrum of chromosomal aberrations in seven MDC1^{-/-} and six MDC1 control (five MDC1^{+/+} and one MDC1^{+/-}) cultures of CD43⁺ splenocytes incubated with concanavalin A (2.5 μ g/mL) for 72 hr. Number of aberrations for each mouse is calculated as (number of aberrations/number of metaphases analyzed)*100. Columns represent average of six

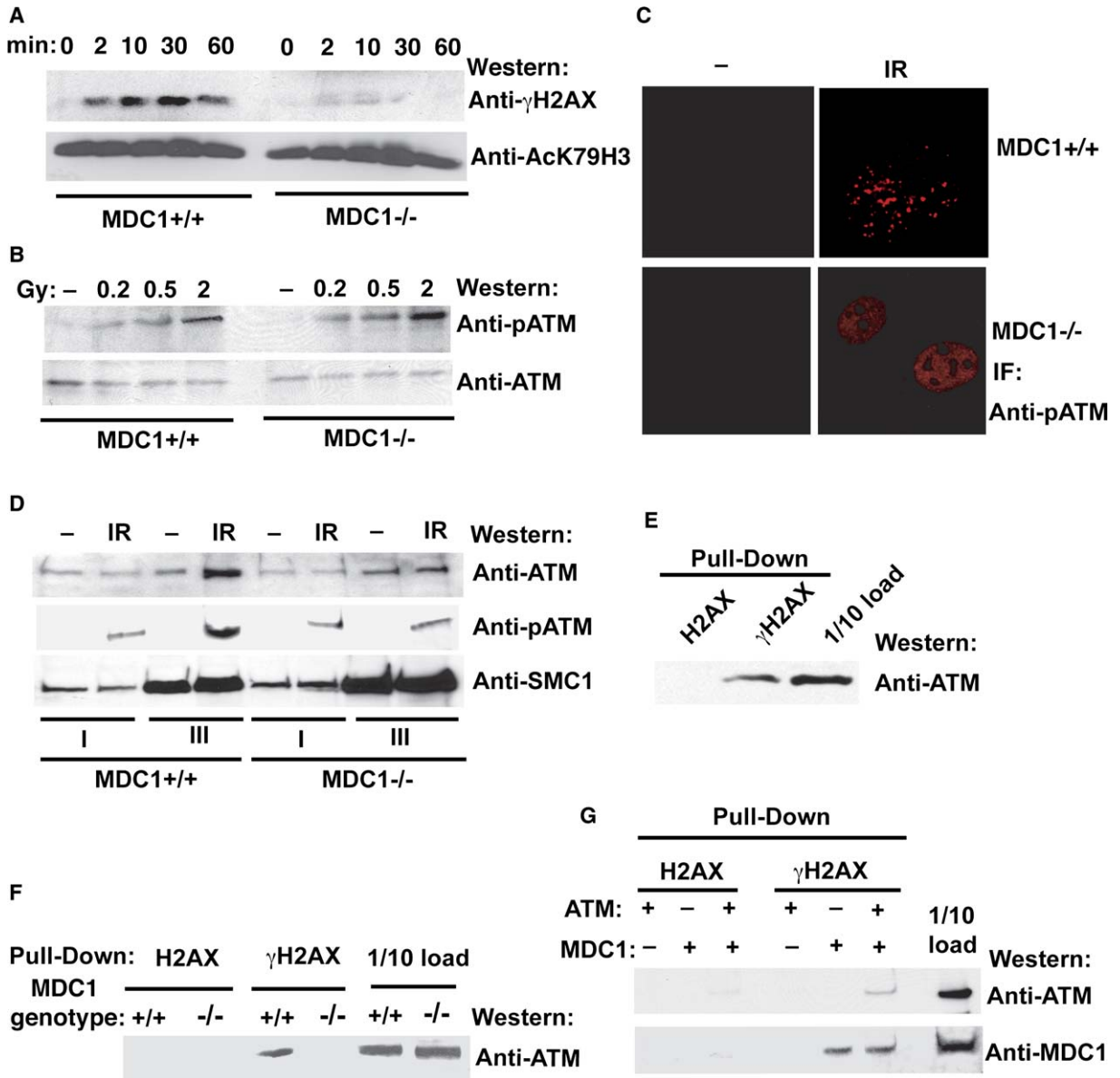


Figure 4. MDC1 Regulates H2AX Phosphorylation and Mediates the Interaction between ATM and γ H2AX

(A) Immortalized MDC1^{+/+} and MDC1^{-/-} MEFs were mock treated or irradiated (1 Gy), and cells were harvested after the indicated time. Histones were extracted from immortalized MDC1^{+/+} and MDC1^{-/-} MEFs and blotted with anti- γ H2AX antibodies. As a loading control, cells extracts were also immunoblotted with anti-Acetyl-K79H3 antibodies.

(B) Immortalized MDC1^{+/+} and MDC1^{-/-} MEFs were mock treated or irradiated at indicated doses, and ATM phosphorylation was detected by immunoblotting with anti-phospho-S1981 ATM antibodies (upper panel). Immunoblotting with anti-ATM antibodies was included as loading control (lower panel).

(C) Immortalized MDC1^{+/+} and MDC1^{-/-} MEFs were mock treated or irradiated (1 Gy). One hour later, cells were fixed and stained with anti-phospho-S1981 ATM antibodies.

(D) Immortalized MDC1^{+/+} and MDC1^{-/-} MEFs were mock treated or irradiated (10 Gy). Thirty minutes later, cells were fractionated as described in the [Experimental Procedures](#). Soluble fraction (fraction I) and chromatin-enriched fraction (fraction III) were blotted with anti-phospho-S1981 ATM, anti-ATM, or anti-SMC1 antibodies as a control.

(E and F) Phosphorylated or control H2AX peptides coupled to sepharose were incubated with cell extracts from 293T cells (E) or MEFs from MDC1^{+/+} or MDC1^{-/-} mice (F). Proteins associated with these peptides were eluted and subjected to immunoblotting using anti-ATM antibodies. Ten percent of cell lysates were loaded as a control.

(G) Phosphorylated or control H2AX peptides coupled to sepharose were incubated with 200 ng purified ATM, MDC1, or ATM plus MDC1. Proteins bound to peptides were then eluted and blotted with anti-ATM or anti-MDC1 antibodies.

(168 metaphase spreads) or seven (240 metaphase spreads) mice for control and knockout, respectively. (F) Examples of chromosomal aberrations in MDC1^{-/-} splenocyte metaphases. Metaphase spreads were hybridized with a telomere Cy3-labeled PNA probe and counterstained with DAPI.

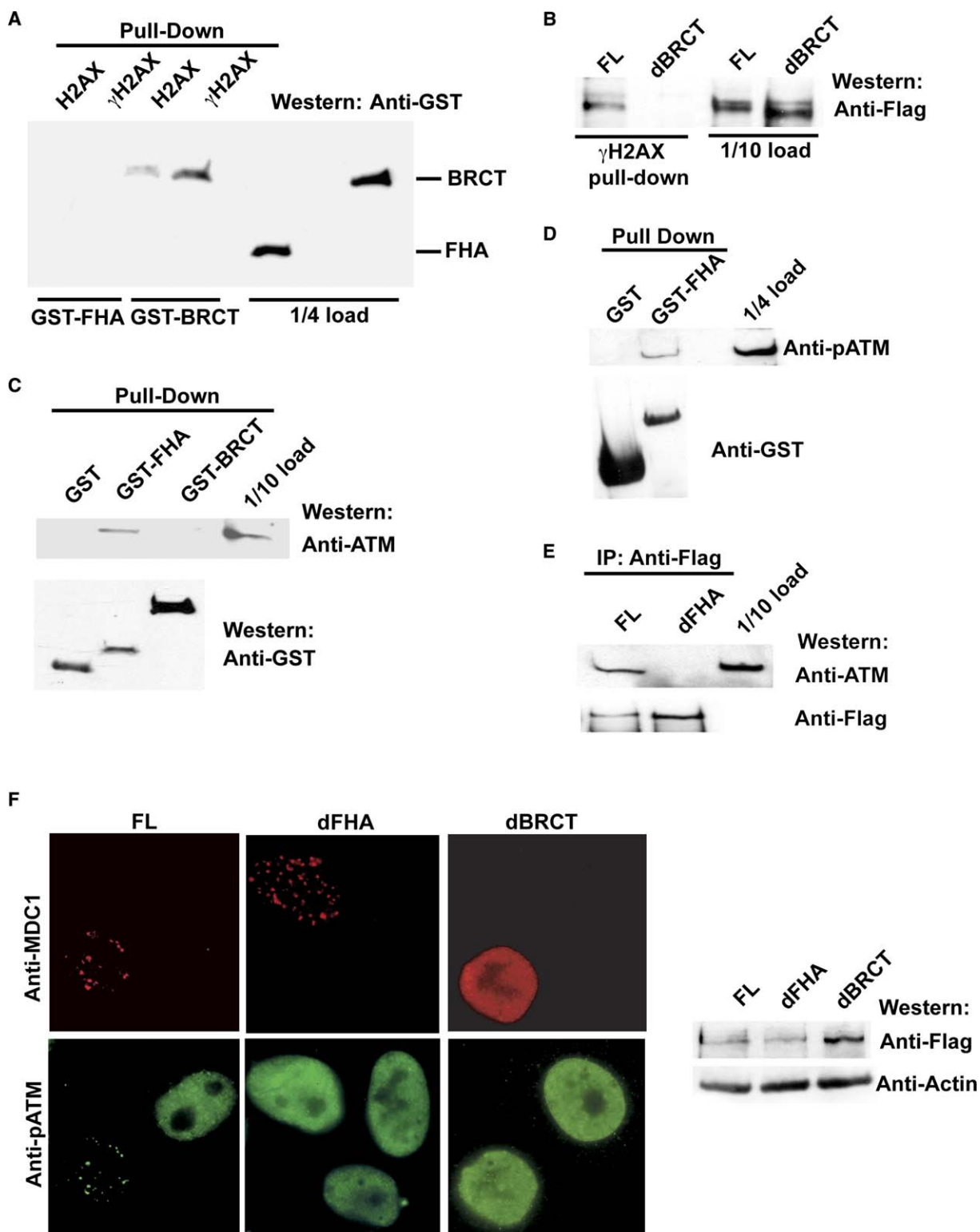


Figure 5. MDC1 Interacts with γ H2AX and ATM through Its BRCT Domain and FHA Domain, Respectively

(A) Phosphorylated or control H2AX peptides were incubated with purified GST-FHA or GST-BRCT domain of MDC1. GST-fusion proteins associated with peptides were detected by immunoblotting with anti-GST antibodies.

(B) 293T cells were transfected with Flag-tagged full-length MDC1 (FL) or MDC1 deleted of the BRCT domain (dBRCT). Cells were then lysed, and cell lysates were incubated with phosphorylated or control H2AX peptides. Proteins associated with these peptides were eluted and subjected to immunoblotting using anti-Flag antibodies.

(C) MDC1 interacts with ATM through its FHA domain. Cell extracts were incubated with GST, GST-FHA, and GST-BRCT domain of MDC1. Proteins bound to GST, GST-FHA, or GST-BRCT were eluted and immunoblotted with anti-ATM antibodies (upper panel). GST-fusion proteins used in pull-down assay were blotted with anti-GST antibodies (lower panel).

ATM. Since the FHA domain of MDC1 has been shown to interact with the M/R/N complex (Goldberg et al., 2003; Xu and Stern, 2003), it is conceivable that the MDC1-ATM interaction might be mediated by the M/R/N complex. However, as shown in Figure S6, the MDC1 FHA domain interacted with ATM in the absence of full-length NBS1, since this interaction still occurs normally in cells deficient of full-length of NBS1. In addition, MDC1 coimmunoprecipitated with ATM in the absence of full-length NBS1 in vivo (Figure S7). In fact, the MDC1 FHA domain interacts with purified ATM in vitro (Figure 5D), suggesting a direct interaction between the MDC1 FHA domain and ATM. Deletion of the FHA domain of MDC1 abolished the MDC1-ATM interaction in vivo (Figure 5E), again confirming that the FHA domain of MDC1 is required for the MDC1-ATM interaction in vivo.

Based on these observations, we speculated that MDC1 might accumulate ATM near the sites of DNA damage through its FHA and BRCT domains, which interact with ATM and γ H2AX, respectively. To test if this is the case, we reconstituted MDC1^{-/-} cells with full-length MDC1 or MDC1 deleted of the FHA (dFHA) or BRCT domain (dBRCT). Reconstitution of wild-type MDC1 restored phospho-ATM foci formation (Figure 5F). MDC1 deleted of the BRCT domain failed to localize to nuclear foci, since it no longer associates with γ H2AX. This MDC1 mutant also could not restore phospho-ATM foci formation. Interestingly, MDC1 with a deletion of FHA domain (dFHA) itself formed foci following DNA damage, since this mutant still retains its ability to interact with γ H2AX. However, this MDC1dFHA mutant still failed to restore phospho-ATM foci formation. This is likely due to the failure of this dFHA mutant to interact with ATM. These results suggest that, through its functional domains, MDC1 facilitates the accumulation of ATM near the sites of DNA damage. By doing this, MDC1 enhances the ATM-dependent H2AX phosphorylation surrounding DNA breaks and therefore amplifies DNA damage signals.

Long-range phosphorylation of H2AX surrounding DNA breaks has been proposed to modulate chromatin structure and facilitate the accumulation of DNA damage-response factors. Early studies suggest that H2AX is not required for the initial recruitment of other checkpoint proteins but is critical for the retention or the accumulation of these checkpoint proteins following DNA damage (Celeste et al. [2003] and data not shown). In agreement with early studies of MDC1 using siRNAs, we failed to observe any damage-induced foci formation for NBS1, 53BP1, and BRCA1 (Figure S8). Based on our hypothesis that MDC1 functions together with H2AX, we would expect that the initial recruitment of these checkpoint proteins would still occur in MDC1^{-/-} cells but that they would fail to accumulate near the sites of DNA breaks. In order to test this possibility, we introduced DSBs using a laser-dissecting microscope. MEFs

treated with laser scissors were then allowed to recover for 5 min. H2AX was phosphorylated along the laser path (Figure 6A). Both NBS1 and 53BP1 redistributed to the path of laser scissor both in wild-type and MDC1^{-/-} cells (Figure 6A), suggesting that the initial recruitment of NBS1 and 53BP1 occurs normally in the absence of MDC1. Four hours after the laser scissor treatment, NBS1 and 53BP1 foci remain along the laser path in wild-type cells, suggesting the accumulation of these factors at the sites of DNA damage (Figure 6A). In contrast, NBS1 and 53BP1 staining was dispersed in MDC1^{-/-} cells, suggesting a failure of accumulation of these checkpoint proteins near the sites of DNA damage (Figure 6A). These results are consistent with previous studies in which MDC1 was downregulated using siRNAs (Bekker-Jensen et al., 2005; Lukas et al., 2004), suggesting that MDC1, like H2AX, is critical for the accumulation of checkpoint proteins near the sites of DNA damage.

In MDC1-deficient cells, we observed the failure of ATM accumulation around the sites of DNA damage. In addition, the retention of downstream ATM substrates near the sites of DNA damage is also abolished in MDC1-deficient cells. This should affect the signal transduction events mediated by ATM, since ATM would then have difficulties accessing its substrates near the sites of DNA damage. We observed defective phosphorylation of NBS1, Chk1, and Chk2 in MDC1^{-/-} cells following 1 Gy of IR (Figure 6B). Similarly, we also observed defective NBS1 and Chk1 phosphorylation in H2AX^{-/-} cells, albeit not as severe as that in MDC1-deficient cells (Figure 6C). Defective ATM signaling observed in H2AX^{-/-} and MDC1^{-/-} cells would also lead to defects in DNA damage checkpoint activation. As expected, both H2AX^{-/-} and MDC1^{-/-} cells displayed the defective G2/M checkpoint (Figures 6D and 6E). These results suggest that failure to amplify ATM signaling in H2AX^{-/-} and MDC1^{-/-} cells is responsible for the DNA repair defects and genomic instability observed in these cells.

To confirm that it is the failure of ATM accumulation that results in defective checkpoint activation, we used siRNA to knock down endogenous MDC1 in human cells and then reconstituted these cells with siRNA-resistant wild-type MDC1 or MDC1 with the deletion of the FHA domain (MDC1dFHA). Although reconstitution of wild-type siRNA-resistant MDC1 rescued the G2/M checkpoint following MDC1 siRNA transfection, MDC1dFHA failed to do so (Figure 6F). Thus, we have demonstrated that the MDC1-dependent accumulation of ATM near the sites of DNA breaks is critical for the amplification of DNA damage signals and proper checkpoint controls following DNA damage.

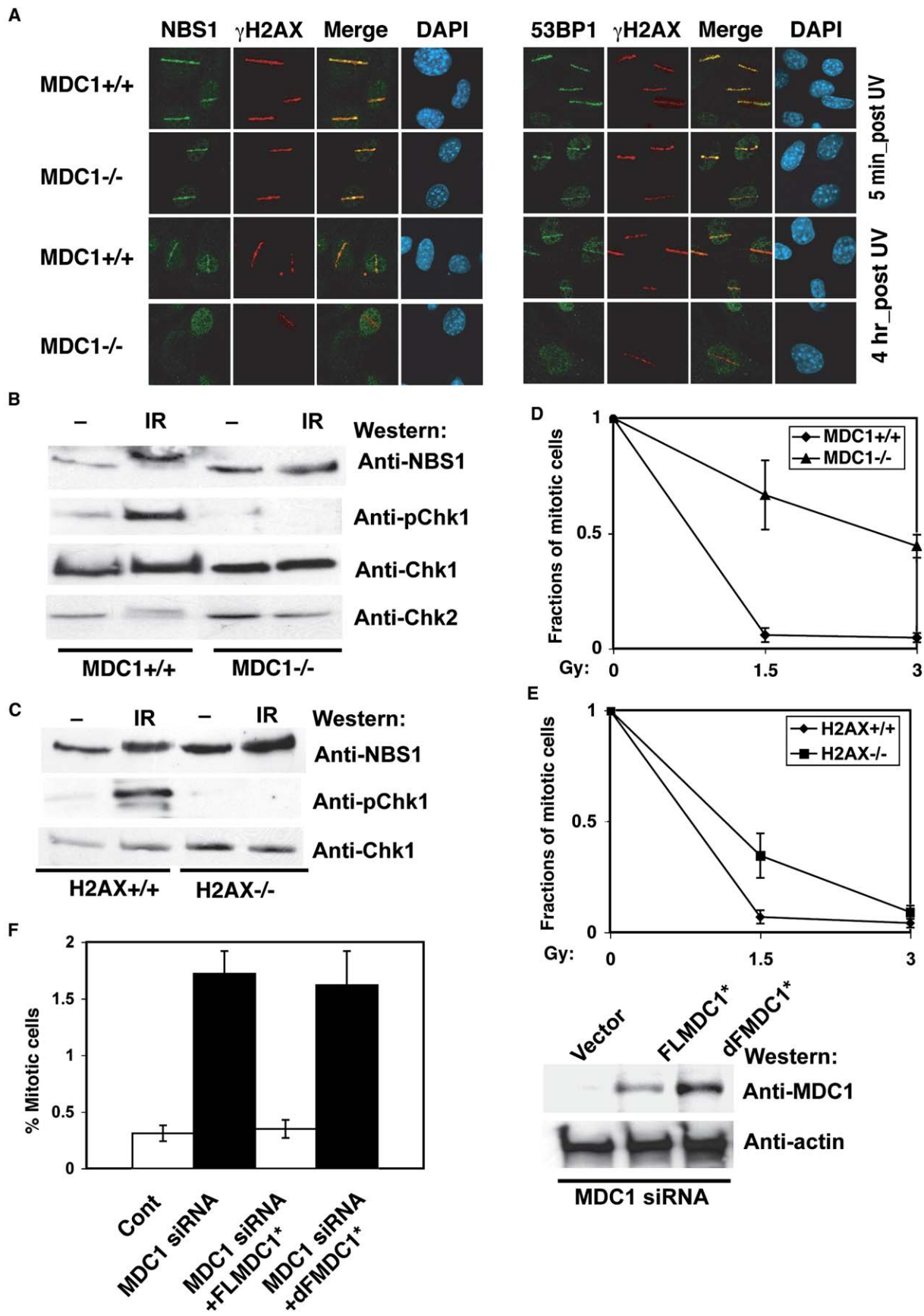
Discussion

The generation of MDC1 knockout mice has provided convincing evidence that MDC1 maintains genomic

(D) Beads bound with GST or GST-FHA domain of MDC1 were incubated with purified ATM. Proteins bound to beads were then eluted and blotted with anti-ATM or anti-GST antibodies.

(E) Flag-tagged full-length (FL) MDC1 or MDC1 with a deletion of the FHA domain (dFHA) was expressed in 293T cells. Immunoprecipitations were performed using anti-Flag antibodies, followed by immunoblotting with anti-ATM or anti-Flag antibodies.

(F) MDC1-deficient cells were reconstituted with full-length MDC1 or MDC1 with a deletion of its FHA (dFHA) or BRCT domain (dBRCT). Cells were irradiated (1 Gy) and stained with anti-phospho-ATM or anti-MDC1 antibodies. Cell lysates were blotted with anti-MDC1 antibodies to show the expression of FL MDC1 or mutant MDC1.



stability in vivo. MDC1^{-/-} mice show significant genomic instability, exemplified by various spontaneous chromosomal aberrations in splenocytes and MEFs. In addition, MDC1^{-/-} mice show growth retardation, male infertility, defective class switching, and increased radiation sensitivity. These phenotypes are almost identical to those of H2AX^{-/-} mice, suggesting that H2AX and MDC1 are close partners in the DNA damage-response pathway. Indeed, we observed reduced H2AX phosphorylation in MDC1-deficient cells. Similar to H2AX^{-/-} cells, we also observed normal initial recruitment but failure of accumulation of DNA damage checkpoint proteins around the sites of DNA damage.

Based on the data presented in this study, we propose the following model for MDC1 function in DNA damage response: following DNA damage, ATM is activated through autophosphorylation at Ser1981 and then recruited to the sites of DNA damage. This initial activation and recruitment of ATM does not require MDC1. MDC1 functions after these initial steps. We believe that ATM, H2AX, and MDC1 form a positive feedback loop, with MDC1 acting as a bridge between ATM and H2AX (Figure 7). Following the initial phosphorylation of H2AX at the sites of DNA breaks (which occurs independently of MDC1), the MDC1-dependent positive feedback loop leads to the accumulation of active ATM near the sites of DNA damage and results in the rapid expansion of H2AX phosphorylation over megabase regions surrounding DSBs. In addition, MDC1 and phospho-H2AX are also responsible for the accumulation of other checkpoint proteins near the sites of DNA damage. By aiding the accumulation of both ATM kinase and its various substrates near the sites of DNA damage, MDC1 helps ATM to phosphorylate its downstream substrates and ensures the proper control of various DNA damage responses. Based on this model, MDC1 is a true mediator of DNA damage checkpoints, facilitating the amplification and transduction of DNA damage signals.

Our findings that H2AX phosphorylation requires MDC1 are consistent with one previous report (Stewart et al., 2003) but conflict with several other reports (Goldberg et al., 2003; Peng and Chen, 2003; Xu and Stern, 2003), which show normal γ H2AX staining in cells transfected with MDC1 siRNA. These discrepancies are probably due to the extent of MDC1 knockdown by siRNA. Overall, our results support a positive role of MDC1 in promoting H2AX phosphorylation, which agrees with the kinetics of H2AX phosphorylation in MDC1-deficient cells. Of course, the binding of MDC1 to γ H2AX could also have a second role, namely protecting H2AX from dephosphorylation. Both of these functions could con-

tribute to the rapid and sustained expansion of H2AX phosphorylation in wild-type cells.

Following DNA damage, the initial phosphorylation of H2AX still occurs in MDC1^{-/-} cells, suggesting that the initial recruitment of ATM occurs even in the absence of MDC1. Recent studies suggest an important role of the M/R/N complex as a DNA damage sensor (Carson et al., 2003; Difilippantonio et al., 2005; Horejsi et al., 2004; Lee and Paull, 2005; Uziel et al., 2003). The M/R/N complex, through the C terminus of NBS1, could be responsible for the optimal activation and initial recruitment of ATM to DSBs (Falck et al., 2005; Lee and Paull, 2005; You et al., 2005). This initial recruitment of ATM by the M/R/N complex, and probably DNA-PK recruitment by Ku, could result in the initial H2AX phosphorylation. MDC1 then binds phosphorylated H2AX and retains activated ATM on chromatin regions near DSBs, which leads to further expansion of H2AX phosphorylation in these regions adjacent to DSBs. Therefore, there appear to be at least two steps involved in ATM signaling, the initial activation and recruitment of ATM to DNA ends (probably regulated by the M/R/N complex) and the subsequent accumulation of ATM on chromatin (regulated by MDC1). The early activation and recruitment of ATM could initiate the signal transduction pathways before the stable MDC1-dependent accumulation of ATM. Diffused active ATM could also transduce signals that do not require MDC1. The fact that MDC1 regulates only parts of ATM's action and is required for the amplification of ATM signals explains why the phenotypes of MDC1 and H2AX knockout mice are milder than those of ATM knockout mice (Barlow et al., 1996; Celeste et al., 2002; Elson et al., 1996; Xu et al., 1996).

In the absence of MDC1, many proteins involved in DNA repair and checkpoint activation, such as NBS1, 53BP1, and BRCA1, fail to accumulate near the sites of DNA damage. Therefore, downstream ATM signaling events, such as the phosphorylation of NBS1, Chk1, and Chk2, are all defective, resulting in an overall deficiency in checkpoint activation and DNA repair. Our results confirm many previous studies obtained by MDC1 knockdown using MDC1 siRNA. However, there are some differences. In contrast to a previous study (Goldberg et al., 2003), we did observe a defect in NBS1 and Chk2 phosphorylation in the absence of MDC1 following DNA damage. It is worth mentioning that lower dose of radiation (1 Gy) was used in our studies than that of previous studies (5–10 Gy). While defective NBS1 phosphorylation is apparent at low dose of radiation (1 Gy), this defect is not obvious at higher doses (5–10 Gy) in MDC1^{-/-} cells (data not shown). This difference probably reflects the nature of MDC1 function in DNA damage

Figure 6. MDC1 Regulates Downstream ATM-Dependent Signaling Events

(A) DSBs were induced by laser scissors in immortalized MDC1^{+/+} and MDC1^{-/-} MEFs. Five minutes or 4 hr later, cells were stained with indicated antibodies.

(B and C) immortalized MDC1^{+/+}, MDC1^{-/-}, H2AX^{+/+}, and H2AX^{-/-} MEFs were mock treated or irradiated (1 Gy). Thirty minutes later, cells were harvested and blotted with indicated antibodies.

(D and E) immortalized MDC1^{+/+}, MDC1^{-/-}, H2AX^{+/+}, and H2AX^{-/-} MEFs were mock treated or irradiated with indicated doses, and, 1 hr later, mitotic populations were evaluated by phospho-H3 staining. The Y axis represents fractions of mitotic populations of irradiated cells compared to those of unirradiated controls. The error bar represents the standard error of results from three independent experiments.

(F) A549 cells were transfected with MDC1 siRNA and reconstituted with siRNA-resistant full-length MDC1 (FLMDC1*) or MDC1 with a deletion of the FHA domain (dFMDC1*). Cells were then mock treated or irradiated (2 Gy). Mitotic populations were evaluated by phospho-H3 staining. The Y axis represents the percentage of mitotic populations. Expression of recombinant proteins was shown at right. The error bar represents the standard error of results from three independent experiments.

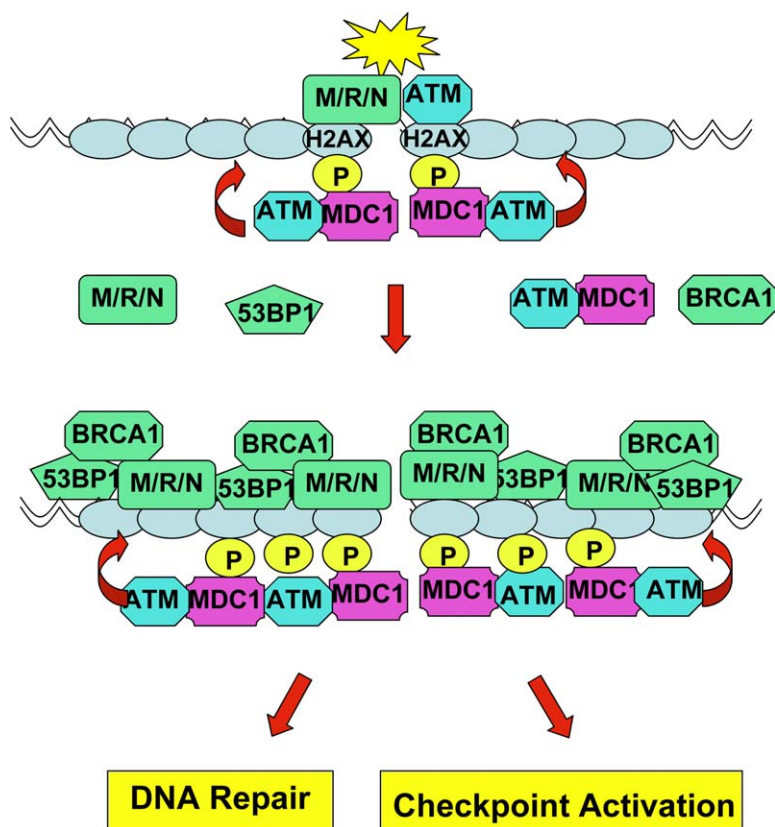


Figure 7. MDC1 Participates in Early DNA Damage Responses

Initial DNA damage signals induce ATM activation and recruitment, which is probably mediated by the M/R/N complex. This initial recruitment of ATM results in early H2AX phosphorylation immediately adjacent to DSBs. Phosphorylated H2AX then binds to MDC1 and accumulates additional MDC1-ATM near the sites of DNA breaks, resulting in the expansion of H2AX phosphorylation to the megabase chromosomal regions surrounding the sites of DNA breaks. This extended H2AX phosphorylation causes changes in chromatin structure and directly or indirectly facilitates the accumulation of downstream ATM substrates at the sites of DNA breaks. ATM then phosphorylates its substrates, resulting in checkpoint activation and DNA repair.

response. While the amplification of ATM signals by MDC1 is clearly required at low, physiological doses of radiation, such an amplification step may be overridden following very high doses of radiation.

It is also interesting that MDC1 mice show IR sensitivity but only a mild class-switching defect, although both processes involve DNA repair. IR could induce many types of DNA damage besides DSBs. Currently, the nature of DNA breaks generated during class switching is not known. From recent publications, 53BP1 seems to play an important role in class switching (Manis et al., 2004; Ward et al., 2004). Therefore, it is possible that different DNA damage repair proteins may be differentially involved in various DNA repair processes. Clearly, further investigation is needed to answer this important question.

In conclusion, we now understand mechanistically how MDC1 may serve as a mediator of DNA damage checkpoints. The data presented here demonstrate that MDC1 amplifies the ATM signaling pathway by accumulating active ATM to areas adjacent to the sites of DNA damage and so promotes the expansion of H2AX phosphorylation. Our studies provide new mechanistic insight into the early events in DNA damage signal transduction and reveal the physiological function of MDC1 in maintaining genomic stability.

Experimental Procedures

Generation of Mice and MEFs

ES clone (OST441263) was obtained from Lexicon Genetics and injected into C57BL/6 blastocysts to generate chimeric mice. The chi-

meras were then crossed with C57BL/6 females, and heterozygous mice with successful germline transmission of the targeted allele were used to generate MDC1^{-/-} mice. MEFs were obtained from E14.5 embryos by a standard procedure. Immortalized MEFs were generated according to standard protocol.

Histone Extractions

Cells were lysed with lysis buffer (0.5% NP40, 100 mM NaCl, 50 mM Tris, 1 mM EDTA). Cell lysates were then centrifuged, and histones were extracted by resuspending pellets with 0.2 N HCl. The HCl extracts were centrifuged, and the supernatants (histone extracts) were then neutralized with 1 N NaOH and blotted with anti-γH2AX antibodies.

Peptide Pull-Down Assay

H2AX peptides (CKATQASQEY) with non-phospho- or phospho-S139 were synthesized by the Mayo protein core facility. Peptides (2 mg) were coupled to Sepharose beads using the SulfoLink Kit (Pierce). Coupled peptides were then incubated with cell lysates to perform pull-down assay. Proteins bound to peptides were then eluted with SDS sample buffer, separated with SDS-PAGE, and blotted with indicated antibodies. For in vitro binding of H2AX peptides with purified ATM or MDC1, biotinylated H2AX peptides (200 μg) were bound to streptavidin-agarose and incubated with 200 ng purified ATM, MDC1, or combinations of ATM and MDC1. Proteins bound to peptides were then eluted with 2 mM biotin, separated with SDS-PAGE, and blotted with anti-ATM or anti-MDC1 antibodies. Purified ATM was obtained as previously described (Lee and Paull, 2005). Purified MDC1 was produced by infecting insect cells (SF21) with baculovirus encoding His-Flag-tagged MDC1.

Chromatin Fractionation Assay

Chromatin fractionation assay was performed according to protocols provided by Dr. A. Nussenzweig. Briefly, MEFs were lysed in buffer I (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.05% NP40, and protease and phosphatase inhibitors) for 5 min on ice. Cell lysates were centrifuged at 3500 rpm for 5 min at 4°C. The

supernatants were collected (fraction I). The precipitates were washed once with buffer I (fraction II), then extracted with buffer II (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, and protease and phosphatase inhibitors) on ice for 20 min. The extracts were centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were collected as the chromatin-enriched fractions (fraction III).

Generation of DSBs by Using Laser Scissors

The laser scissors approach has been performed as described previously (Celeste et al., 2003).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and eight supplemental figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/21/2/187/DC1/>.

Acknowledgments

We thank Dr. John H.J. Petrini (Memorial Sloan Kettering Cancer Center) for providing anti-mouse NBS1 antibodies and Dr. Yosef Shiloh (Tel Aviv University) for providing anti-mouse ATM antibodies. We thank Jamie Wood for providing suggestions and proofreading of this manuscript. This work is supported by grants from National Institute of Health (NIH RO1 CA89239 and CA92312 to J.C.). J.C. is a recipient of DOD breast cancer career development award (DAMD17-02-1-0472). Z.L. is a recipient of DOD breast cancer fellowship award (DAMD17-03-1-0610).

Received: September 29, 2005

Revised: November 18, 2005

Accepted: November 29, 2005

Published: January 19, 2006

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